APPENDIX B
Submitted Written Public Comments*

(Additional Written Public Comments from the November 15, 2004 Meeting may be found on the 911 Environmental Action Website: http://911ea.org)

***THE FOLLOWING PUBLIC COMMENTS WERE RECEIVED AT THE EXPERT TECHNICAL PANEL REVIEW MEETING #8. NOTE, THE MEETING IS NOT A PUBLIC HEARING TO HEAR TESTIMONY, BUT RATHER A TECHNICAL MEETING FOR EXPERT PANEL MEMBER DISCUSSIONS WITH TIME SET ASIDE TO HEAR COMMENTS FROM THE PUBLIC ON DISCUSSION TOPICS.**

STATEMENT OF ROBERT GULACK, UNION STEWARD, U.S. SECURITIES & EXCHANGE COMMISSION, AT THE EPA TECHNICAL REVIEW PANEL

FOR IMMEDIATE RELEASE
November 15, 2004

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On November 2, 2004, Manhattan voted more than four to one to free itself from this White House and this EPA, who have, for more than three years, forced the innocent children of this city to inhale and ingest the life-threatening contamination left behind by the terrorists. As surveys have previously shown, the citizens of New York have lost all trust in the EPA. But Manhattan was not allowed to rescue itself. New York has been left to the ghoulish mercies of the White House and the grisly gang at EPA that works the wicked will of the White House.

We in New York remain bound to the stake of history, and, against our will as expressed by our overwhelming ballots, must ready ourselves to receive lash after lethal lash from the President and the Governor and the Mayor who continue to betray us and our children. And now, after helping to stall the clean-up effort for three years, after helping to stall this panel for eight months so that the scientific facts could be hidden from the voters, Dr. Gilman is preparing to leave us. Before he goes, I have some questions for him.

I will make these questions brief and non-rhetorical, and so provide Dr. Gilman with one last chance to wash some of the blood off his hands. I am going to be referring to an article entitled, "Critics Say Unclear EPA Role At Ground Zero May Undermine Cleanup," by the Clean Air Report via InsideEPA.com, vol. 15, no. 23, from November 4, 2004, which may be read at environmentalnewsstand.com. That article quotes an EPA agency source as saying, "EPA has repeatedly clashed with some New York city officials regarding responsibility for cleaning up WTC contamination. Several sources say city health officials fear that findings of high lead levels -- whether associated with the WTC attacks or not -- would trigger city requirements to remove the lead and could even lead to possible lawsuits from residents. Gilman has met with city officials several times -- most recently in October -- to try to reach an agreement with city officials, the agency source says."

My question is simply this: is this article accurate? Has New York City opposed testing for lead because New York City's political leadership prefers to avoid lawsuits, even at the cost of damaging the brains of helpless children? Have you, Dr. Gilman, met with city officials several times, including as recently as October, in an attempt to reach agreement with these recalcitrant city officials? Is it true that a major source of resistance to the testing sought by the New York community is to be found in the machinations, behind closed-doors, of Mayor Bloomberg and his appointees?

Dr. Gilman, the people of New York have the right to a yes or no answer on this question before you leave this room. If you, Dr. Gilman, have, in fact, gone "several times" to New York and seen your earnest entreaties rebuffed, is it not now time for you to declare, on the record, that you are not the problem in this area, that the real objections have come from New York? To whom in New York have you spoken? What contaminants have been discussed? What plan

does the EPA have for overcoming the resistance of the New York City leadership and proceeding with testing?

Yesterday's *New York Post* put it this way: "Like the EPA, the city is also balking at a further cleanup. Fearing the multi-million dollar expense and possible lawsuits, city officials are pushing the EPA not to test for lead in its search for more hazardous materials, said members of the EPA expert panel who asked not to be identified." Evidently, this is an open secret, well-known to everyone in New York except the voters. Furthermore, we just heard this morning that city and federal buildings are refusing to prove background samples.

I have a few other questions. Every member of this panel, including the co-chair, Dr. Lioy, understands the importance of prompt testing in Brooklyn. You alone, Dr. Gilman, continue to hold out against evidence and logic. The EPA continues to act as if you – the EPA employee on this panel – were the sole member of this panel. The EPA continues to refuse to schedule testing in Brooklyn. When you convene a panel, and set it to work for eight months, and listen only to your own employee, you make the panel process a fraud. Has someone told you to oppose testing in Brooklyn? Who was it?

Speaking of Dr. Lioy, he requested long ago that the EPA assemble, from all the relevant national, state, and city agencies, a complete legal analysis of the conditions under which those national, state, and city agencies can respond to requests for testing from individual residents and employees. For example, once an individual employee makes a request for OSHA testing, can EPA walk in the door with OSHA? For example, why does this panel not request Governor Pataki to give the authority to city safety officials to respond to requests from residents and employees? Why does EPA continue to refuse to coordinate a thorough and professional group memo from the legal staffs of the relevant agencies, spelling out in writing what can and cannot be done? Has someone told you not to push for such a memo? Who was it? How can a rational sampling plan be published for public comment, without providing such a legal analysis as to what is and isn't possible? We all heard Dr. Gilman this morning continue to refer only to the EPA's powers, taken in isolation. The issue is not the legal powers of the EPA, all by itself. The issue is: what are the unified powers, taken together, of EPA, OSHA, and the relevant state and city agencies, working together as a group to provide an adequate and complete response to this situation?

Fifty community groups, including Community Board 1 and Congressman Nadler, and many substantial unions representing many tens of thousands of workers, have now endorsed the Seven Principles for cleaning up New York submitted in October to the EPA and this panel. Not a single community group or union has opposed the Seven Principles. No one opposes them, except for a Texan in the White House and you, Dr. Gilman. Will you, Dr. Gilman, now admit that, whether or not you personally agree with the Seven Principles, the Seven Principles, do, in fact, express the will of the people of the affected areas? Will you, Dr. Gilman, recommend as you leave that the EPA cease to insinuate that the Seven Principles do not have the support of the community?

Decades ago, a haughty dictator announced that he had lost confidence in the people of his country, and that the people could only regain his confidence by redoubled efforts. A poet of

that nation responded that, if, indeed, the people of that country had lost the confidence of their government, the government had no alternative – it had to dissolve the people and elect another one. That is the situation we now find ourselves in. The EPA has lost confidence in the people of New York. Instead of trying to clean up this three-year-old mess, the EPA is now busying itself trying to dissolve the people of New York and elect another one. They are running around trying to find some community group – somewhere – that they can fool into endorsing the EPA's fraudulent testing proposal. This is a waste of time and money. The delay will only cause further illness. No one in New York is going to endorse the EPA's approach, which we all understand is calculated to find nothing and thus allow EPA to abandon this process. As White House e-mails have made clear from September 2001 onwards, for you, this has always been a political game. For us, it has always been a matter of life and death.

[In response to this statement, Dr. Gilman denied that anyone had told him to oppose testing in Brooklyn or to refuse to prepare a comprehensive legal memo. He also denied the accuracy of the InsideEPA article, but refused to comment upon the article in the *New York Post*.]

I have written comments but something was said this morning that made a light go off [on?] and that was about 'averaging.' It reminded me of the days right after September 11 when we had a meeting at Stuyvesant. Juan Gonzalez had just published his article 'Toxic Zone' which I had brought along. George Thurston [NYU] said, 'Put that away; that's yellow journalism. Those levels are all spikes.'

Also Lung Chi Chen, when he talked about asbestos, said that in general there was no problem downtown. I asked him about the levels at Stuyvesant [2.4 million s/sqcm via ultrasonication] and he said they were anomalies.

When it comes to ascertaining the truth about what people are being exposed to, "averaging" is the kiss of death. It has been used to wash away inconvenient data that don't conform with the norm. And here it is in this sampling plan. [I'll say more about this next meeting]

Written comments:

1. When EPA announced thier cleanup in May 02, we asked them, "What about Brooklyn?" EPA said, "We're considering that for a possible Phase Two of this program." Phase Two never happened.

As we discuss this sampling proposal, once again we ask, "What about Brooklyn?" Once again EPA says, "We're considering that for a possible Phase Two of this program." That's in spite of EPA's statement on page twelve: "The collapse of the WTC towers produced many tons of dust and this dust spread over a wide area of Manhattan and beyond."

You will understand some skepticism on the part of the public about a possible Phase Two of the program. Particularly when Phase One is going to spend time, energy and money on sampling desktops and countertops which are among the most frequently cleaned surfaces in any apartment.

2. Page 23 mentions testing only for fibers greater than five microns.

This is another issue that refuses to die. It's been raised particularly with respect to asbestos but similar principles may apply to other materials as well.

About the dangers of fibers less than five microns, I recommend again that the panel consult the memos of EPA's Dr. Cate Jenkins, particularly those of February 21, 2003 and July 4, 2003. Also Dr. Lippmann chaired the ATSDR panel in October 02 in which several experts observed that the attribute of asbestos that made it dangerous might not be length so much as an aspect ratio of 3:1.

Finally, Dr. Meeker, didn't you say in your presentation that most of the particles you found were three microns?

Meeker: Yes.

3. When EPA comes up with a new testing proposal, it is accustomed to seeking peer review. The need for third party approval extends also into the area of execution of such proposals. This panel has heard powerful testimony from the public on the many and diverse ways in which EPA's contractors bungled the testing and cleanup first time around. And Andrew Schneider's article of January 14, 2002, discusses EPA's use of instruments that were twenty years old. For every fiber of asbestos that EPA found, independent contractors, using up-to-date equipment, found nine.

Is there any reason to think EPA will perform better now? As time goes on, EPA's need grows only stronger to prove that it is not liable for any failures in the past. And evidence mounts only higher of OUR need to monitor their activity. To quote a current sage who's had almost as great an impact on the English language as he's had on the rest of the world, "Fool me once, shame on.... shame on you. It fool me. We can't get fooled again." [Bush]

4.The reason for the poor rate of participation in the last cleanup was EPA's outreach which was based on the premise that the purpose of the cleanup was to allay people's concern. Then they told people there was no reason for concern. Their flier explicitly stated "We do not expect longterm health consequences" from any WTC dust that might still be lurking in people's homes.

In order to prevent history from repeating itself, this community should have input into the outreach as we have had into the panel process. Dr. Gilman, may we have that input?

Gilman: Yes. That's why I came to the community meeting.

Orkin: Including into the wording?

Gilman: Yes.

Jenna Orkin
World Trade Center Environmental Organization

Testimony at EPA meeting 11/15/04
Caroline Martin
Family Association of Tribeca East

I am part of a thinly represented group before this panel – building owners.

As far as owners are concerned, testing must be attached to cleaning.

I have been informed by my building's attorney that there appears to be an obligation on the part of owners to inform prospective purchasers about known building contaminants – especially lead.

Thus if as a building owner you volunteer your building for testing, anything deleterious that is found will have to be reported. This is why cleaning must be integrally related to testing.

In short – testing should not take place in a vacuum!

For building owners, concerns include damage to property during cleaning, diminished real estate values, and the fact that if you find unsafe levels of COPCs behind my fridge – you will do nothing about it; but I will have to report it to potential buyers. The same would apply to COPCs present but my failure to have slag wool.

I am not sure that under these circumstances I would want to open my building for testing.

TESTIMONY OF SUZANNE Y. MATTEI, NYC EXECUTIVE OF SIERRA CLUB
BEFORE THE EPA WTC EXPERT TECHNICAL REVIEW PANEL
NOVEMBER 15, 2004

The Ground Zero community soon will have technical experts to help review the sampling plan that is before you today. In the meantime, these are preliminary comments submitted on behalf of the Sierra Club to present a context for our concerns.

The goal of this project should be to find whatever is out there that may still present a risk to human health from World Trade Center pollution and remove it. The goal should not be to find as little as possible or to do as little as possible. The Panel should consider this goal as it reviews the proposed sampling plan.

We urge the Panel to design the testing program and clean-up triggers so that the program is most likely to result in protection of human health, and we raise four concerns at this time:

(1) The sampling plan (Subdivision E of Section I) states that EPA plans to sample for lead only on hard surfaces, using dust wipe tests. This limited approach does not make sense from a scientific or public health perspective. Lead can easily become embedded in carpets and soft furniture. Because lead dust particles can escape an ordinary vacuum, a contaminated carpet is likely to remain so unless it is professionally abated. This is one of the most likely locations to find WTC lead dust today, a location that presents a special exposure risk to very young children.

Dust wipe sampling of floors and other hard surfaces alone is effective in identifying a hazard if the source of the lead in an apartment is a continuing condition such as lead paint hazards. If the source is WTC dust, however, then dust wipe sampling on frequently cleaned hard surfaces – especially surfaces that are washed – three years after the contamination event is much less likely to reveal its presence. Soft surfaces, in contrast, may well still be harboring WTC lead dust. EPA must not ignore soft surfaces when sampling for lead in an apartment.

(2) We are baffled by the notion that EPA will not use test results from so-called "inaccessible" areas for the cleanup decision, but only to measure the transport of WTC pollution. The two examples that EPA gives for so-called "inaccessible" areas are "behind or on top of cabinets." Such locations are not inaccessible. While people may not visit these areas daily, many people do cleaning once or twice a month that involves moving furniture or placing or removing items from tops of cabinets. A few times a year, people may shift books around in a bookcase. It is not clear why Osama Bin Laden should be allowed to contaminate those surfaces and expose those people. What broadly-accepted public health analysis and regulatory foundation justifies tolerating contamination in the home so long as it is not located on a tabletop or floor?

(3) There are four decisions that must be made after testing –

(A) whether or not a cleanup of that particular location should occur;
(B) whether or not further testing should be done of that building;
(C) whether or not further testing should be done in the surrounding area and
(D) if the site is located near the border of the Phase I testing zone, whether or not testing should be expanded beyond that border.

We object to using three-times background level as the minimum trigger for clean-up or further testing in a building. Osama Bin Laden should not be granted the right to triple the level of pollution in our homes and workplaces. Whether or not this three-times background level would be reasonable as a trigger for further testing in the community or beyond a border also must be carefully examined. We do urge EPA to design the Phase Two testing trigger cautiously. The test buildings will be selected on a volunteer basis; they might be more likely to be cleaner than other buildings. EPA should consider that potential bias in determining the need for further testing in a neighborhood.

(4) This Panel has been struggling with the "signature" issue regarding responsibility for cleanup of a contamination problem. We are concerned by the criteria for "success" in validating the WTC signature, which states that the signature study must be "fully successful in identifying a signature in indoor dust that can be reliably tied to the building collapse." EPA must recognize that it is conducting this sampling program three years after the event. (That is not the community's fault. The community has been clamoring for proper testing throughout this period.) We must assume that some mixing of dust will occur in some, if not many, instances. The "signature" should not be so rigidly defined as to exclude genuine cases of WTC contamination and unfairly burden individual owners with a cleanup problem that rightly should be remedied by the federal government.

We urge the Panel to ensure that any "signature" be defined with ample flexibility to consider the likely mixing of ordinary dust with WTC dust. The standard should not – and probably cannot – be absolute "certainty." A standard of "more likely than not" would be appropriate. Again, it is not the community's fault that so much time has passed since the original polluting event, and the community should not be penalized for any lack of absolute certainty in "signature" identification. The cleanup trigger must be designed to protect the public from further exposure to WTC dust. That must be the primary goal – not absolute certainty of source three years after an event.

We further urge the Panel to consider that the content of WTC dust may well have varied based on deposition distance, since different substances and differently-sized particles have varying abilities to be transported over distance

Finally, we urge that EPA supervise safety during the Deutsche Bank demolition. I spent several years investigating safety hazards that occur in New York City schools when renovation is conducted while classes are in session. Despite promises and declarations of concern for children, I found hazardous conditions in project after project. A public authority was doing the work, yet neither the City DEP nor the City health department were able to control it. There is no reason to believe that the Deutsche Bank demolition will be done more carefully. If EPA does not take control, this community will get hit again by WTC pollution. That should not be tolerated.

We hope to provide more detailed testimony after consulting with technical experts through the partnership process.

Testimony to EPA Expert Panel on WTC Contamination
November15, 2004

Robert L. Jaffe, Ph.D.
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http://www.envirolab.com

Discussions conducted by the EPA Expert Panel on WTC Contamination "have led to the concept that a WTC signature exists in dust and that sampling could focus on determining the presence of that signature, as well as the levels of contaminants of potential concern." Although each of the COPC is identified with a specific health risk benchmark value, there are no benchmark values for the **total mixture** of COPCs found in each dust sample. Data is not available for the possible **toxic synergy** of these mixtures. Furthermore, there may be "new" contaminants, not previously listed as a suspect agents.

I propose that an appropriate bio-monitoring assay be employed in order to assess the toxicity and potential long-term health effects of the complete mixture of contaminants in WTC dust in indoor environments. Specifically, I propose the use of the *Tetramitus* Assay. *Tetramitus* is a single cell flagellate which ingests whole particles. Growth inhibition data are generated from flagellate cultures which are exposed to either soluble toxicants or toxic whole particles. Attached to this memorandum is my recent report to the EPA Health Effects Laboratory[1], which documents toxicity tests of flagellates exposed to NIST Standard Reference Materials as well as reference toxicants used in the EPA WTC Mouse Exposure Studies[2]. The dose response for exposure to these toxic particles is illustrated in Figure 1. The linearity of the dose-response regression lines is an important feature of this assay. Because of the high r^2 values(correlation coefficients), single dose determinations can be employed for screening a large number of dust samples.

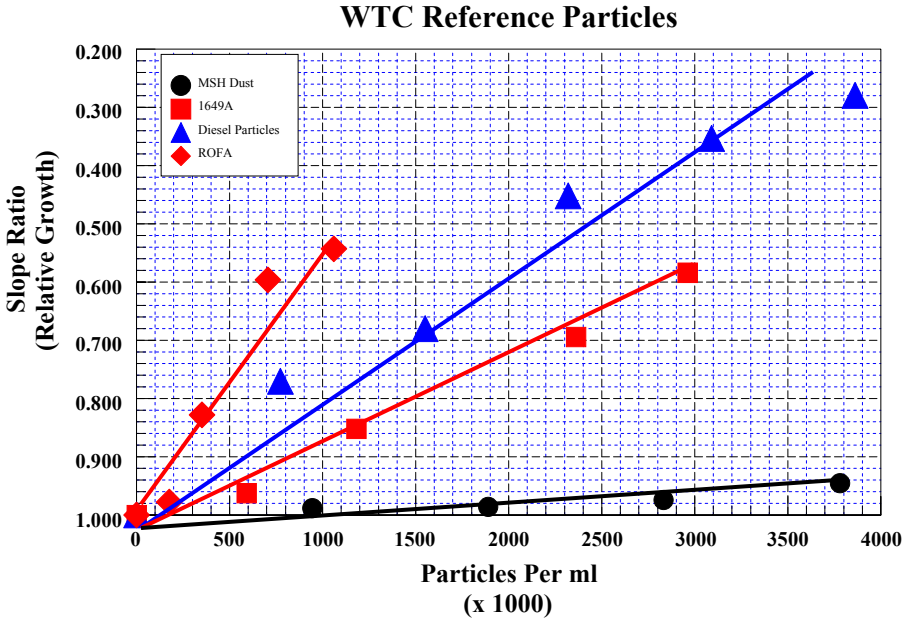


Figure 1. Dose Response of *Tetramitus* flagellates exposed to WTC Reference Samples.

Evidence is presented, in the *Tetramitus* Report to the EPA, which supports the hypothesis that this growth inhibition is associated with DNA damage. Lewitas, et.al,[3] have published values for the mutagenicity of diesel particles, 1649a (Urban Air), and coal tar using the Ames *Salmonella* test. *Tetramitus* also exhibits dose-dependent toxicity to coal tar[4,5]. DNA damage is the postulated first step in the conversion of normal cells to cancer cells. DNA damage also impairs the immune system; thus, compromised individuals are at greater risk for a whole spectrum of infectious diseases.

The inclusion of the *Tetramitus* Assay as one test in a battery of tests would now address the issue of **mixtures**. After demonstration of *Tetramitus* test concordance with COPC test results, the *Tetramitus* Assay could be employed as a cost-effective pre-screening tool to expand the frequency of testing and to extend the geographic limits of the building survey.

References:

1. Jaffe, R.L. (2004) Utility of the *Tetramitus* Assay for the Assessment of Air Quality following Terrorist Attacks. Report to the U.S. Environmental Protection Agency Pulmonary Toxicology Branch, Experimental Toxicology Division, NHEERL Revised Report October 11, 2004

2. (2002) , Stephen H. Gavett, Najna Haykol-Coates, John K. McGee, Jerry W. Highfill, Allen D.Ledbetter and Daniel L. Costa. Toxicological Effects of Fine Particulate Matter Derived from the Destruction of the World Trade Center. United States Environmental Protection Agency, Office of Research and Development, National Health and Environmental Effects Research Laboratory, Research Triangle Park, NC . September, 2002. EPA/600/R-02/028.

3. (1992) J. Lewtas, L.D. Claxton, H.S. Rosenkranz, D. Schuetzle, M. Shelby, H. Matsushita, F.E. Wurgler, F.K. Zimmerman, G. Lofroth, W.E. May, D. Krewski, T. Matsushima, Y. Ohnishi, H.N.G. Gopalan, R. Sarin, and G.C. Becking Design and Implementation of a Collaborative Study of the Mutagenicity of Complex Mixtures in *Salmonella typhimurium*. Mutation Research 276: 3-9)

4. (1995) Jaffe, R.L., Rapid Assay of Cytotoxicity Using Tetramitus Flagellates. Toxicology and Industrial Health 11: 543-558

5. 2000, Jaffe, R.L, The Tetramitus Assay, in Biomonitors and Biomarkers as Indicators of Environmental Change, Ed: Butterworth, F.M., A.Gunatilaka, and M.E. Gonsebatt pp.391- 425.Kluwar Academic/Plenum Publishers (New York)

Utility of the Tetramitus Assay for the Assessment of Air Quality following Terrorist Attacks

Robert L. Jaffe, Ph.D.

Report to the U.S. Environmental Protection Agency
Pulmonary Toxicology Branch
Experimental Toxicology Division, NHEERL
Revised Report October 11, 2004

Environmental Toxicology Laboratory LLC

Utility of the Tetramitus Assay for the Assessment of Air Quality following Terrorist Attacks

The use of the free-swimming, single cell, flagellate, *Tetramitus rostratus* to measure whole particle toxicity, offers a unique solution for the rapid monitoring of air quality following terrorist attacks. Previous *Tetramitus* studies of tap water [(1)], reservoir sites [(2)], and various streams and rivers in the Croton Watershed[(3)] have described the presence of particles, which cause partial or total growth inhibition in *Tetramitus* flagellates. These studies have not been accepted within various regulatory agencies because of the lack of information concerning the molecular mechanism, which causes this growth inhibition. In spite of the indirect evidence, which supports the hypothesis, that DNA damage is the mode of action (26/27 DNA-damaging agents produced growth inhibition), the *Tetramitus* Assay remains unrecognized as a test to monitor whole particle toxicity and the toxicity of environmental pollutant mixtures.

Recent tests using two National Institute of Standards and Technology (NIST) standard reference materials (SRMs) also produced growth inhibition of *Tetramitus* flagellates. Both coal fly ash (SRM#2689) and diesel particle exhaust (SRM#2975) produced positive dose responses in growing flagellate cultures (Figure 1). The EPA recently has classified diesel exhaust as a human carcinogen.

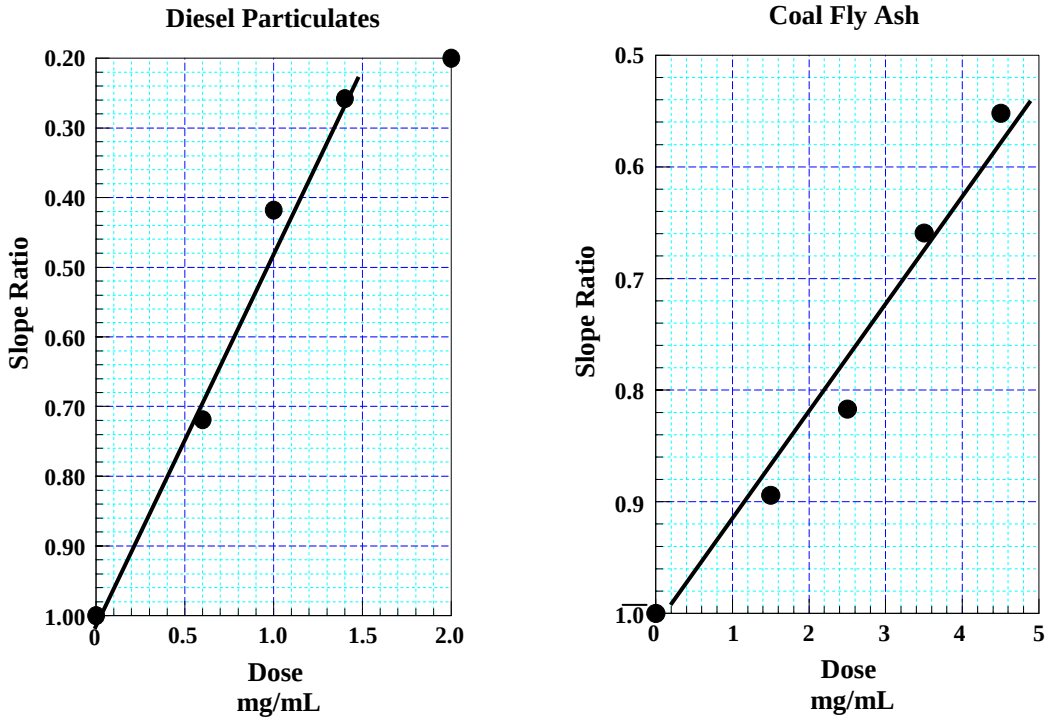


Figure 1. Dose response of *Tetramitus* flagellates exposed to National Institute of Standards and Technology, Coal Fly Ash and Diesel Particulate Matter. Lower Slope Ratios (Relative Growth) indicate increased toxicity.

In order to provide further data for the evaluation of the *Tetramitus* Assay, a study was designed to measure the dose response of growing flagellates to the three reference toxicants described in the EPA study: "Toxicological Effects of Fine Particulate Matter Derived from the Destruction of the World Trade Center"[4]. The three reference Toxicants, obtained from the EPA National Health Effects Laboratory, Pulmonary Toxicology Branch, in Research Triangle Park, NC were: ROFA (residual oil fly ash), SRM 1649a (Washington DC Total Suspended Particles from NIST) and Mt. St. Helens Dust. These reference toxicants were tested in the *Tetramitus* Growth Inhibition Assay. ROFA was the most toxic, 1649a also was toxic, and Mt. St. Helens Dust produced a very low toxicity response in both the *Tetramitus* Assay and the EPA mouse exposure studies. .

Materials and Methods

The three reference toxicants were sent to ETL by Dr. Stephen H. Gavett, Pulmonary Toxicology Branch, U.S. Environmental Protection Agency, Research Triangle Park, NC.
The diesel particulate matter (SRM-2975) was obtained from the National Institute of Standards and Technology (NIST). Portions of the samples were transferred to 20 mL liquid scintillation vials and weighed. Aliquots of MS-1 buffer (see Appendix I) were added in order to prepare suspensions at concentrations of 3 mg/mL. The initial test of ROFA at 3 mg/mL resulted in flagellate cell death at all but the lowest dose tested. A subsequent test series was conducted using a ROFA concentration of 1 mg/mL. The pH of the ROFA and NIST 1649a suspensions was below 6.6 as judged by the yellow color of the phenol red indicator in the MS-1 buffer. Addition of 40 µl of 0.1N $NaCHO_3$ raised the pH to 7.4 –7.6. Three doses of an adjusted MS-1 buffer were tested to see if this caused any change in *Tetramitus* flagellate growth. Table 1 contains the regression data for the MS-1 and the three $NaHCO_3$ adjusted-MS-1 dose growth curves. The mean slope value was 0.08358. The coefficient of Variance was 0.460. The 99% confidence interval was (0.08246 – 0.08470).

Table 1. Regression Data for $NaHCO_3$ –Adjusted MS-1

(Time Intervals = 0 – 22.02 –26.14 Hours)

Dose	Slope	r^2	Slope Ratio
MS-1	.08359	.9999	1.0
100 µL	.08304	.9999	.993
300 µL	.08377	.9998	1.002
500 µL	.08392	.9996	1.004

The suspensions were parsed into 14 mL Falcon culture tubes according to the dilution schedule, shown in Appendix 1. 50 µl aliquots of concentrated Kp and 50 µL of *Tetramitus* Seed culture were then added to each dose tube. The tubes were incubated at 30°C in a rotary shaker @180 RPM. Lotus 123 spread sheets were set up and 200µl aliquots were withdrawn at indicated times, added to the Folin Wu dilution tubes, and counted in the Coulter ZM Counter in order to obtain flagellate growth data for each dose and time sampled. Regression data and the slope ratios for each dose culture were automatically computed with the use of the embedded Lotus-123 macro commands (see Appendix 1).

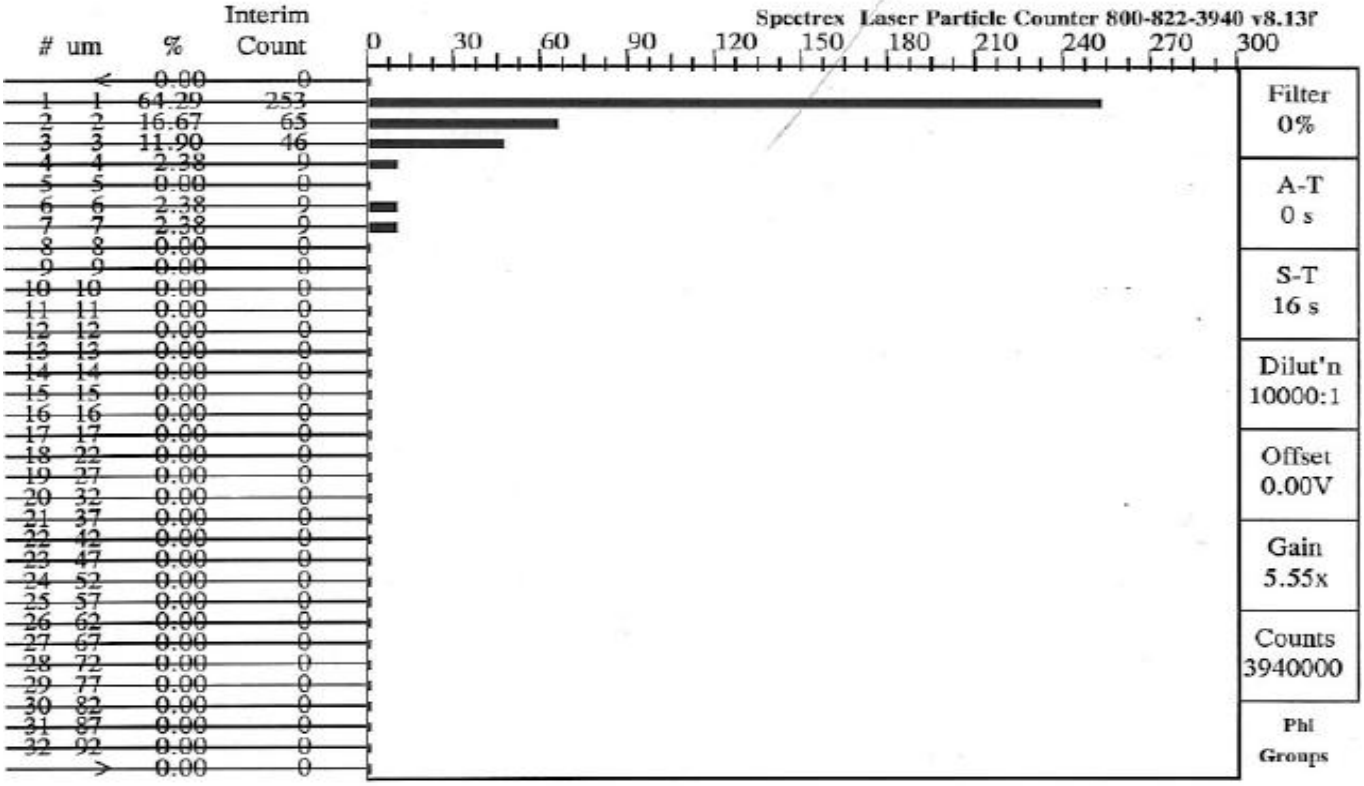
Particle concentrations and size distributions were obtained with a Spectrex 2000PC Laser Particle Counter (http://www.spectrex.com). A 10µL aliquot of each particle suspension was transferred to 100mL of Distilled water with a predetermined background count below 50. The particle suspensions were subjected to vigorous agitation with a vortex genie mixer prior to transfer of the 10µL aliquot. The 1/10,000 dilution factor was entered into the Spectrex data program. None of the three WTC Reference Toxicants were size-fractionated. The Diesel Particulate Suspension was passed through a 47mm Savur Filter – Pore size 25µm (GE-Osmonics. DFP25WPP12) in order to exclude larger particles. The Laser Particle Scans for each of the four samples are shown below (Figures 2-5)

Results

Size Distributions and Particle Concentrations

Spectrex Laser Particle Scans of the four particle suspensions are shown in Figures 2-5.
The particle concentrations for each dose tested are calculated from the undiluted particle suspensions and are listed in Tables 2-5.

Residual Oil Fly Ash (ROFA)#2
1.0 mg/mL
LPS 1/10,000 (10 ul/100mL)
27-Sept-03
BKG =41



#	um	%	Interim Count
<		0.00	0
1	1	64.29	253
2	2	16.67	65
3	3	11.90	46
4	4	2.38	9
5	5	0.00	0
6	6	2.38	9
7	7	2.38	9
8	8	0.00	0
9	9	0.00	0
10	10	0.00	0
11	11	0.00	0
12	12	0.00	0
13	13	0.00	0
14	14	0.00	0
15	15	0.00	0
16	16	0.00	0
17	17	0.00	0
18	22	0.00	0
19	27	0.00	0
20	32	0.00	0
21	37	0.00	0
22	42	0.00	0
23	47	0.00	0
24	52	0.00	0
25	57	0.00	0
26	62	0.00	0
27	67	0.00	0
28	72	0.00	0
29	77	0.00	0
30	82	0.00	0
31	87	0.00	0
32	92	0.00	0
>		0.00	0

Phi	Size	Total counts /cc	Counts percent	Surface area percent	Volume percent
10	1-2	2,532,857.14	64.29%	13.43%	0.60%
9	2-4	1,125,714.29	28.57%	36.32%	11.50%
8	4-8	281,428.57	7.14%	50.25%	87.90%
7	8-16	0.00	0.00%	0.00%	0.00%
6	16-31	0.00	0.00%	0.00%	0.00%
5	31-63	0.00	0.00%	0.00%	0.00%
4	63-128	0.00	0.00%	0.00%	0.00%

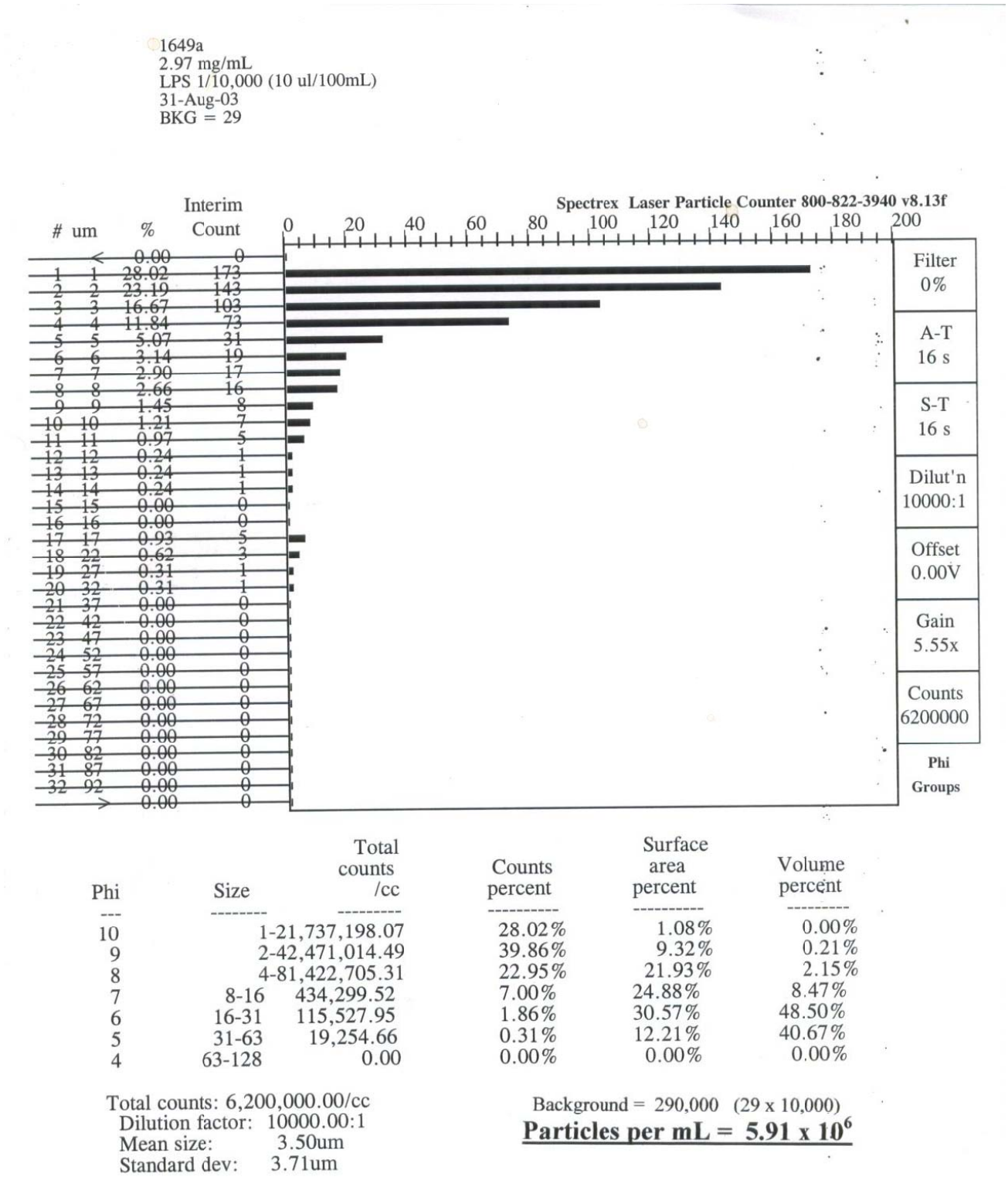
Total counts: 3,940,000.00/cc
Dilution factor: 10000.00:1
Mean size: 1.74um
Standard dev: 1.33um

Background = 410,000 (41 x 10,000)
Particles per mL = 3.53×10^6

Page 1 of 2

Sample taken on September 27, 2003 at 21:53

Figure 2. Particle Size Distribution and Concentration for ROFA Suspension (1.0 mg/mL).



Phi	Size	Total counts /cc	Counts percent	Surface area percent	Volume percent
10	1-2	1,737,198.07	28.02%	1.08%	0.00%
9	2-4	2,471,014.49	39.86%	9.32%	0.21%
8	4-8	1,422,705.31	22.95%	21.93%	2.15%
7	8-16	434,299.52	7.00%	24.88%	8.47%
6	16-31	115,527.95	1.86%	30.57%	48.50%
5	31-63	19,254.66	0.31%	12.21%	40.67%
4	63-128	0.00	0.00%	0.00%	0.00%

Total counts: 6,200,000.00/cc
Dilution factor: 10000.00:1
Mean size: 3.50um
Standard dev: 3.71um

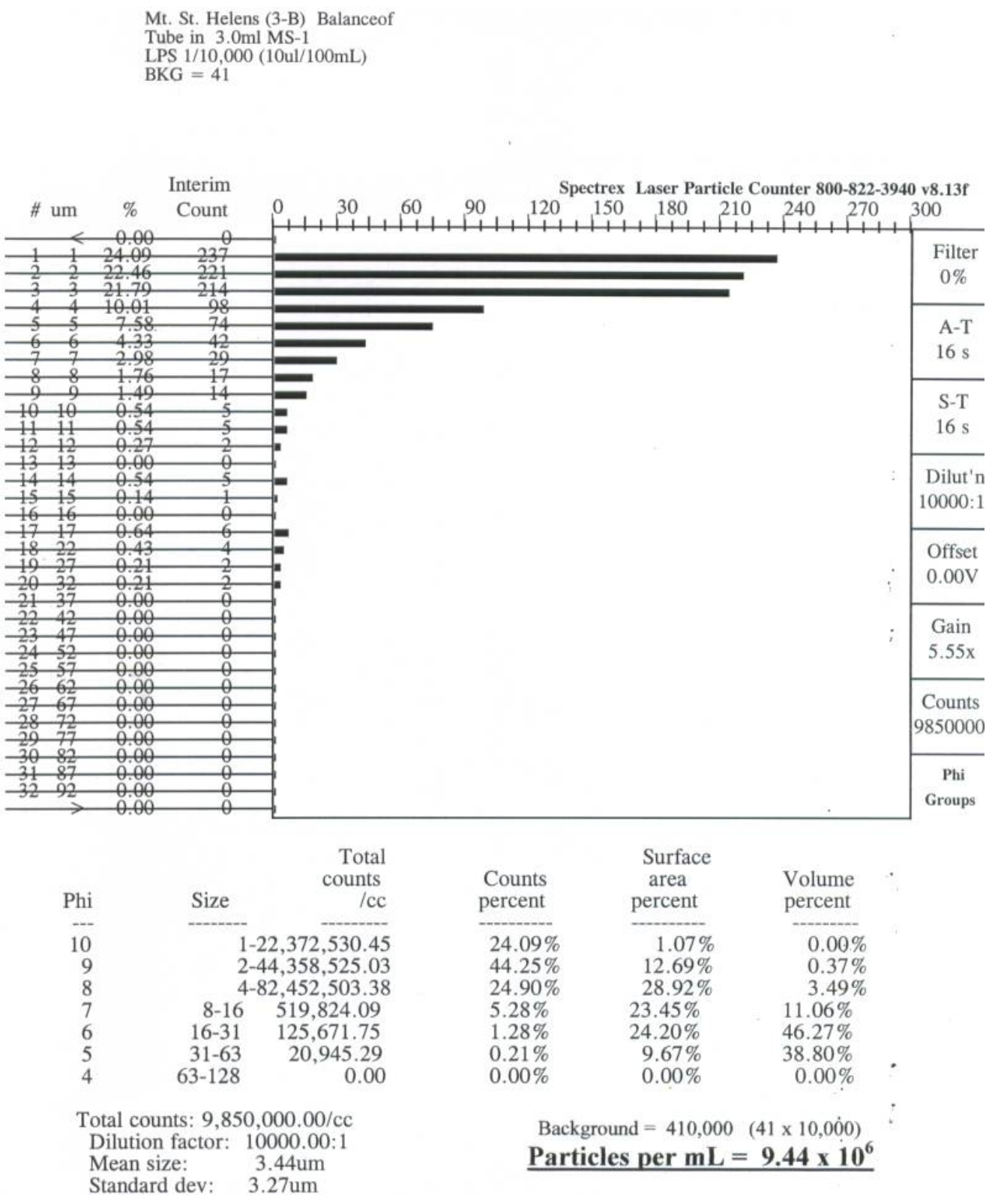
Background = 290,000 (29 x 10,000)

Particles per mL = 5.91×10^6

Page 1 of 2

Sample taken on August 28, 2003 at 01:01

Figure 3. Particle Size Distribution and Concentration for NIST 1649a Suspension (2.97 mg/mL).



#	um	%	Interim Count
<		0.00	0
1	1	24.09	237
2	2	22.46	221
3	3	21.79	214
4	4	10.01	98
5	5	7.58	74
6	6	4.33	42
7	7	2.98	29
8	8	1.76	17
9	9	1.49	14
10	10	0.54	5
11	11	0.54	5
12	12	0.27	2
13	13	0.00	0
14	14	0.54	5
15	15	0.14	1
16	16	0.00	0
17	17	0.64	6
18	22	0.43	4
19	27	0.21	2
20	32	0.21	2
21	37	0.00	0
22	42	0.00	0
23	47	0.00	0
24	52	0.00	0
25	57	0.00	0
26	62	0.00	0
27	67	0.00	0
28	72	0.00	0
29	77	0.00	0
30	82	0.00	0
31	87	0.00	0
32	92	0.00	0
>		0.00	0

Phi	Size	Total counts /cc	Counts percent	Surface area percent	Volume percent
10	1	22,372,530.45	24.09%	1.07%	0.00%
9	2	44,358,525.03	44.25%	12.69%	0.37%
8	4	82,452,503.38	24.90%	28.92%	3.49%
7	8-16	519,824.09	5.28%	23.45%	11.06%
6	16-31	125,671.75	1.28%	24.20%	46.27%
5	31-63	20,945.29	0.21%	9.67%	38.80%
4	63-128	0.00	0.00%	0.00%	0.00%

Total counts: 9,850,000.00/cc
Dilution factor: 10000.00:1
Mean size: 3.44um
Standard dev: 3.27um

Background = 410,000 (41 x 10,000)

Particles per mL = 9.44×10^6

Figure 4. Particle Size Distribution and Concentration for Mt. St. Helens Dust Suspension.

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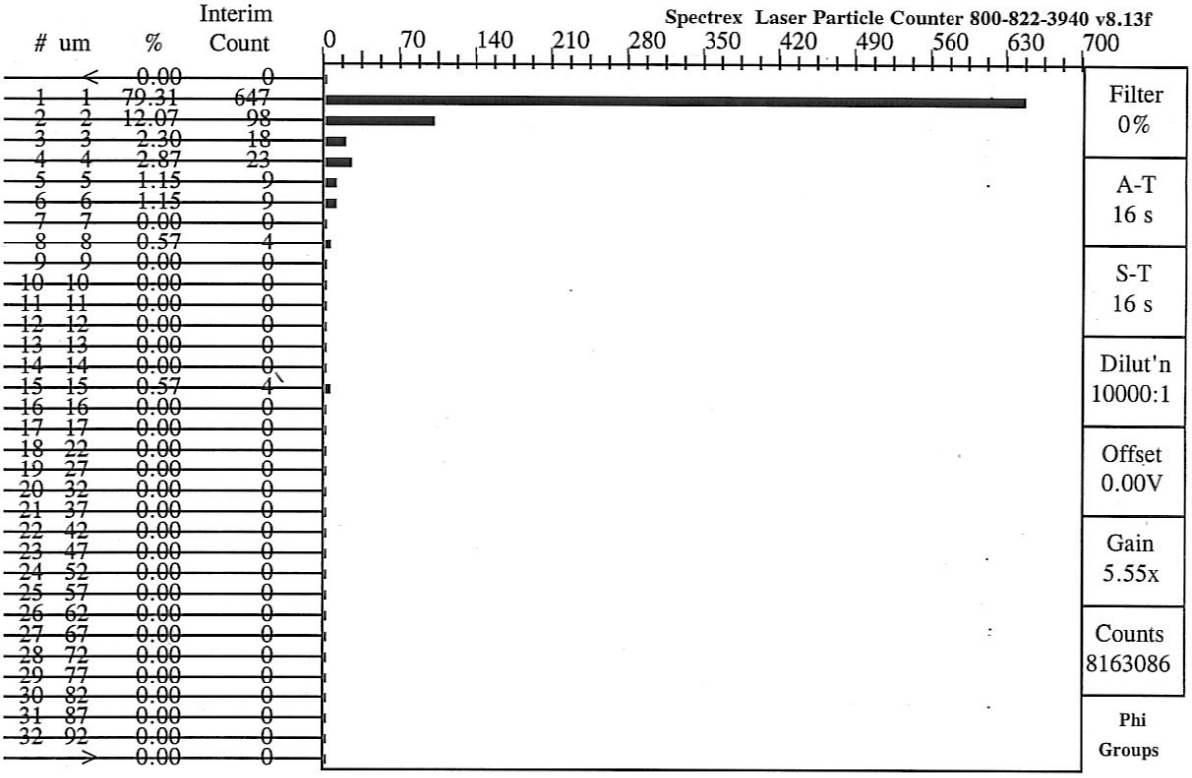
NIST Diesel Particulate
2 mg/mL (16mg/8mL) SAVUR
FILTRATE
LPS 1/10,000 (10ul/100mL)
BKG = 43



Phi	Size	Total counts /cc	Counts percent	Surface area percent	Volume percent
10	1-	26,474,171.43	79.31%	18.42%	0.23%
9	2-	41,172,857.14	14.37%	16.02%	1.09%
8	4-8	422,228.57	5.17%	26.97%	8.45%
7	8-16	93,828.57	1.15%	38.58%	90.24%
6	16-31	0.00	0.00%	0.00%	0.00%
5	31-63	0.00	0.00%	0.00%	0.00%
4	63-128	0.00	0.00%	0.00%	0.00%

Total counts: 8,163,085.71/cc
Dilution factor: 10000.00:1
Mean size: 1.48um
Standard dev: 1.46um

Background = 430,000 (43 x 10,000)

Particles per mL = 7.73×10^6

Page 1 of 2

Sample taken on September 1, 2003 at 22:13

Figure 5. Particle Size Distribution and Concentration for Diesel Particulate Suspension (2.0 mg/mL)

Tetramitus Toxicity Assays

Figure 6 shows the dose response for the three EPA reference toxicants and the NIST Diesel Particulates. Tables 2-5 show the regression data, particle concentrations, and mg/mL for the three EPA Reference Toxicants and the Diesel Particulate Matter. The dose-growth curves for each particle suspension are shown in Figures 7-10.

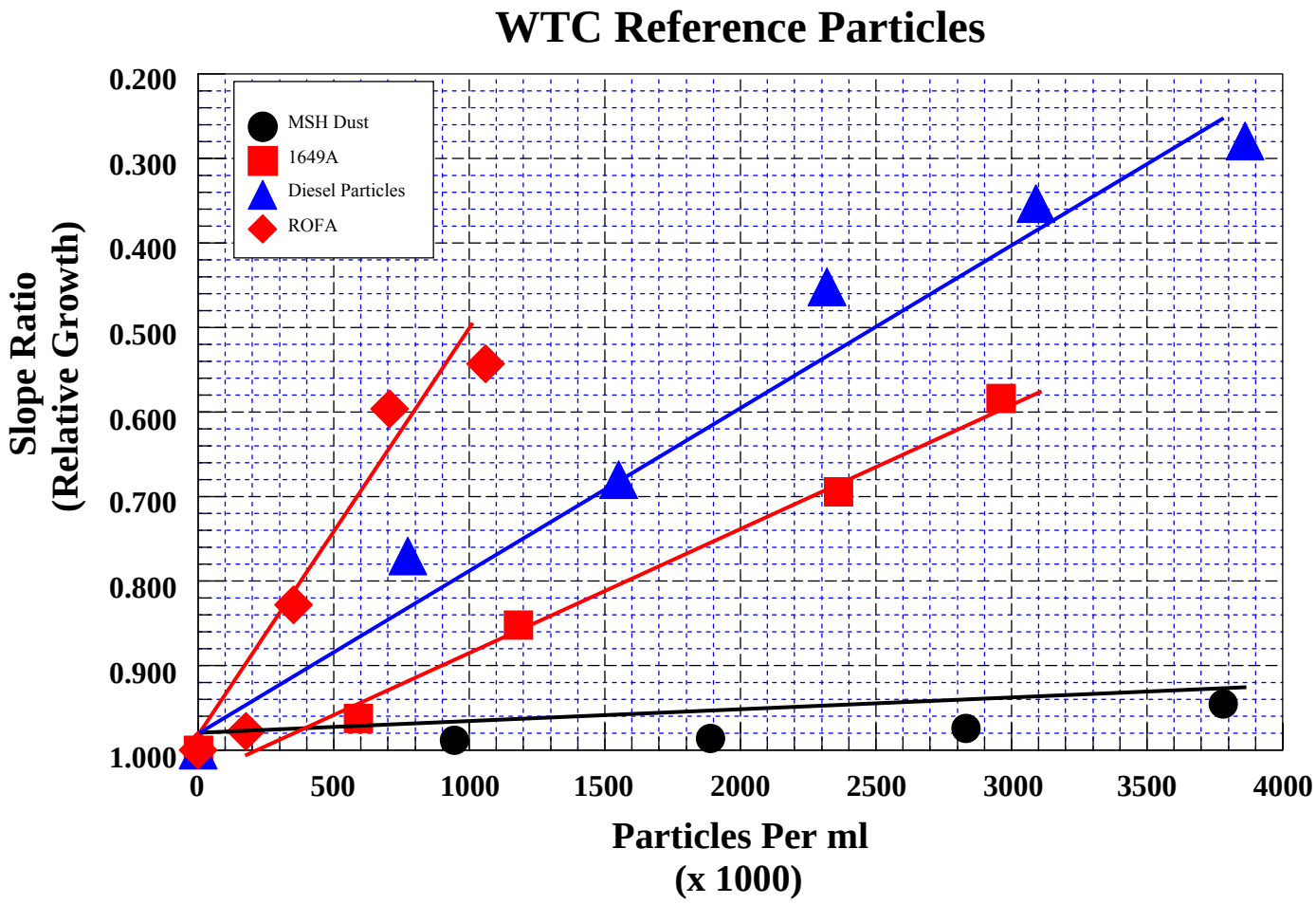


Figure 6. Dose Response of *Tetramitus* flagellates exposed to different concentrations of EPA Reference Samples, used in the WTC Mouse Toxicity Study. At ROFA doses above 1.06 x 10^6 particles per ml, flagellate growth was completely inhibited after 25 hours of exposure (See Fig 7).

Table 2. Regression Data for ROFA

(Time Intervals = 0 - 21.35 – 25.37 Hours)

Dose	Particle Concentration	mg/mL	Slope	r^2	Slope Ratio
MS-1	----	----	.08366	.9991	1.0
10 µl	3.53×10^4	0.01	.08426	.9995	1.007
20 µl	7.06×10^4	0.02	.08391	.9998	1.003
50 µl	1.77×10^5	0.05	.08181	.9999	.978
100 µl	3.53×10^5	0.1	.06927	.9999	.828
200 µl	7.06×10^5	0.2	.04985	.9985	.596
300 µl	1.06×10^6	0.3	.04547	.9996	.543
500 µl	1.77×10^6	0.5	.04981	.9566	.595

Table 3.

Regression Data for NIST 1649a

(Time Intervals = 0 – 23.39–33.18 Hours)

Dose	Particle Concentration	mg/mL	Slope	r^2	Slope Ratio
MS-1	----	----	.08206	.9992	1.0
100 µl	5.91×10^5	0.3	.07893	.9999	.962
200 µl	1.18×10^6	0.6	.06993	.9850	.852
300 µl	1.77×10^6	0.9	.06753	.9808	.823
400 µl	2.36×10^6	1.2	.05697	.9534	.694
500 µl	2.96×10^6	1.5	.04794	.8822	.584
700 µl	4.14×10^6	2.1	.05116	.9676	.623
900 µl	5.32×10^6	2.7	.05678	.9391	.692

Table 4.

Regression Data for Mt. St. Helens Dust

(Time Intervals = 0 – 22.72 –32.61 Hours)

Dose	Particle Concentration	mg/mL	Slope	r^2	Slope Ratio
MS-1	----	----	.08286	.9971	1.0
100 µl	9.44×10^5	NA*	.08186	.9991	.988
200 µl	1.89×10^6	NA	.08171	.9989	.986
300 µl	2.83×10^6	NA	.08070	.9997	.974
400 µl	3.78×10^6	NA	.07831	.9998	.945
500 µl	4.72×10^6	NA	.07975	.9997	.963
700 µl	6.61×10^6	NA	.08031	.9963	.969
900 µl	8.50×10^6	NA	.08163	.9999	.985

* The MSH particle suspension was prepared by adding MS-1 buffer to the original sample tube, Thus, the weight was not available (NA).

Table 5.

Regression Data for NIST Diesel Particulate Matter (SRM-2975)

(Time Intervals = 0 – 22.99 – 32.95 Hours)

Dose	Particle Concentration	mg/mL*	Slope	r^2	Slope Ratio
MS-1	----	----	.08303	.9986	1.0
100 µl	7.73×10^5	0.2	.06395	.9999	.770
200 µl	1.55×10^6	0.4	.05644	.9909	.680
300 µl	2.32×10^6	0.6	.03753	.9854	.452
400 µl	3.09×10^6	0.8	.02929	.9996	.353
500 µl	3.86×10^6	1.0	.02319	.9999	.279
700 µl	5.41×10^6	1.4	.01730	.9841	.208
900 µl	6.96×10^6	1.8	.01501	.9830	.181

* Original Suspension filtered through a Savur Filter (pore size = 25 µm)

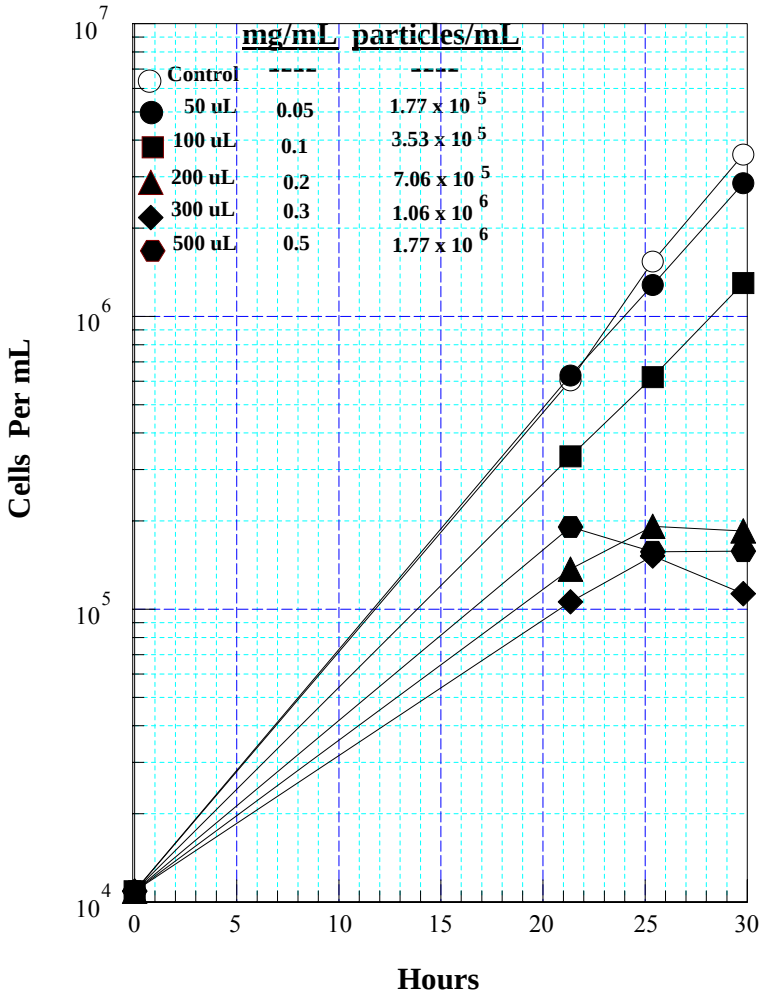


Figure 7. Growth of *Tetramitus* flagellates exposed to ROFA (residual oil fly ash).

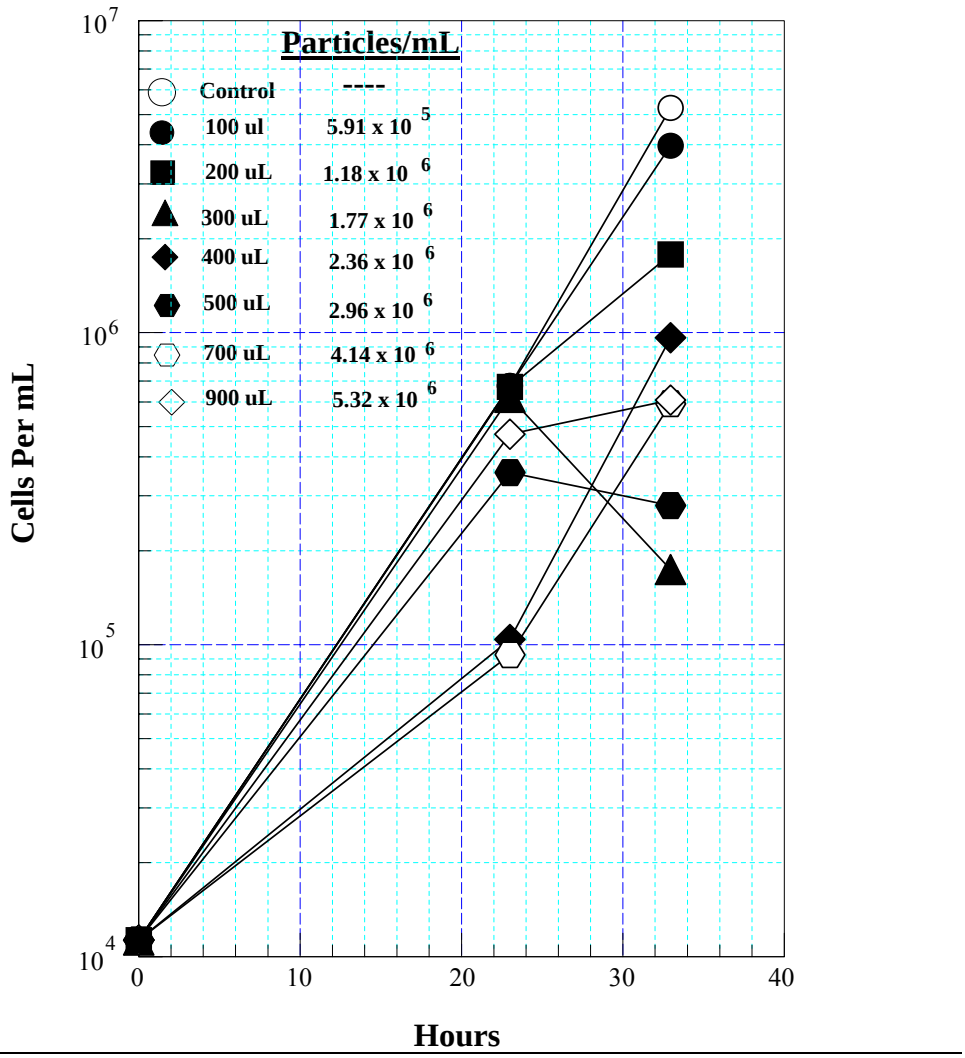


Figure 8. Growth of *Tetramitus* flagellates exposed to NIST 1649a.
Starting with the 500 µL dose, particles settled to the bottom of culture tubes, as judged by visual inspection; thus, the actual concentration of the particles, which remained suspended is uncertain. This may account for the increased growth at the "higher doses" (500, 700 and 900 µL dose tubes).

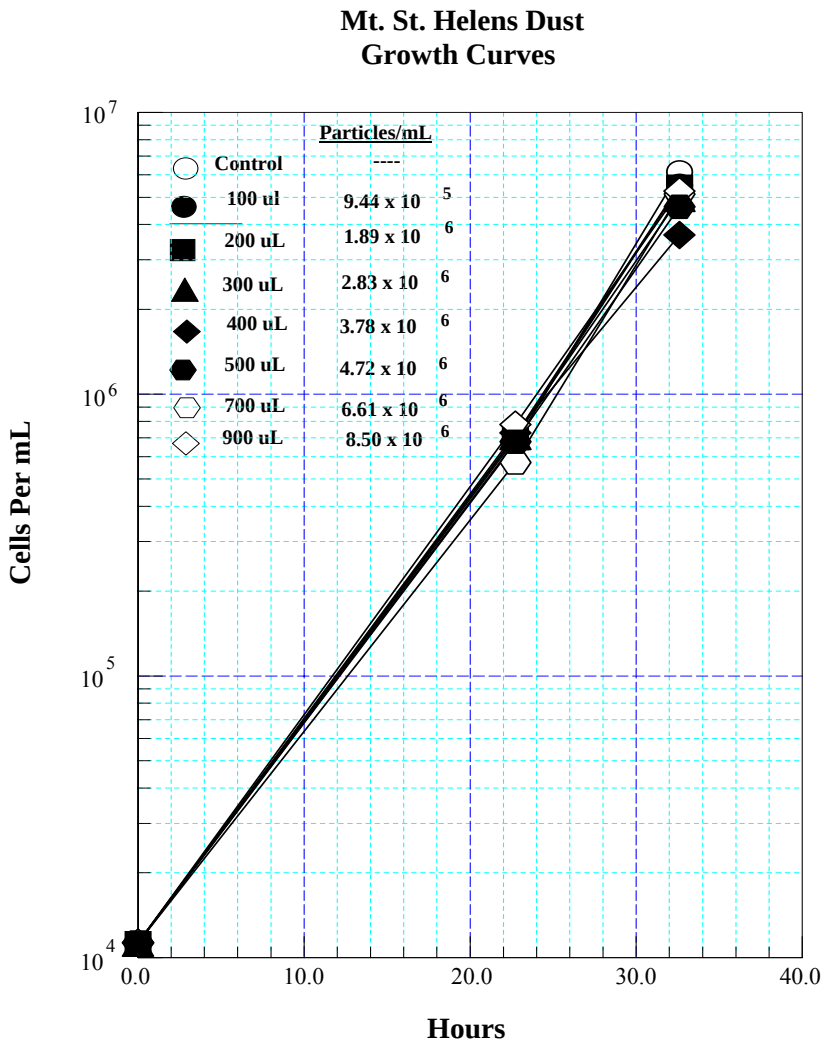


Figure 9. Growth of *Tetramitus* flagellates exposed to Mt. St. Helens Dust.

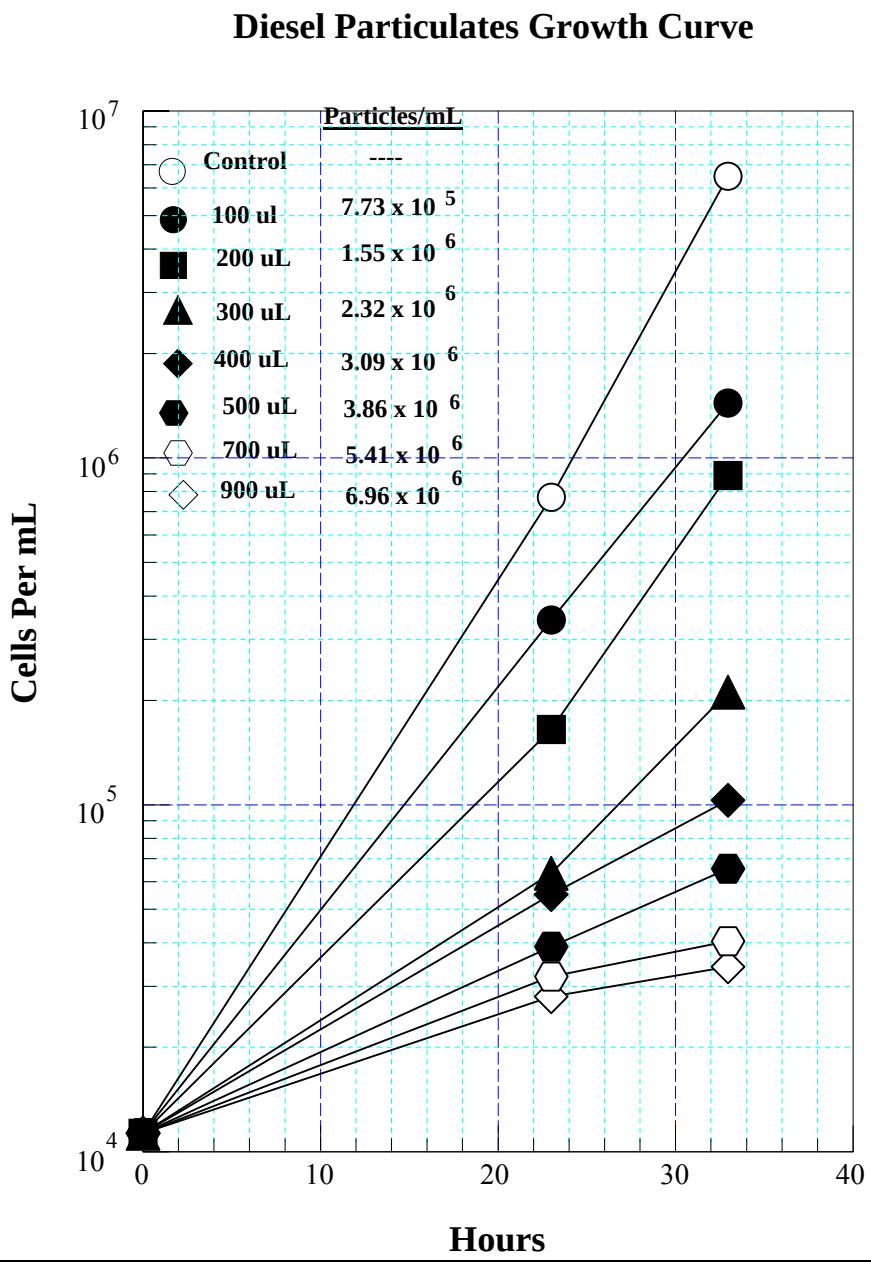


Figure 10. Growth of *Tetramitus* flagellates exposed to Diesel Particulate Matter.

Table 6. Comparison of the Dose Responses (Particle Concentrations) of the 3 WTC Reference Samples and Diesel Particulate Matter.

Sample	NOEC[1] (particles/ml)	LOEC[2] (particles/mL)	TGIC[3] (particles/mL)
ROFA-3	7.06×10^4	1.77×10^5	7.06×10^5
1649a	$< 5.91 \times 10^5$	5.91×10^5	$1.77 - 2.96 \times 10^6$ [4]
MSH	2.83×10^6	3.78×10^6	$> 8.50 \times 10^6$
Diesel	$< 7.73 \times 10^5$	$< 7.73 \times 10^5$	5.41×10^6

(1) No Observable Effect Concentration
(2) Lowest Observable Effect Concentration
(3) Total Growth Inhibition Concentration
(4) This number is an estimate, bearing in mind the settling out of NIST 1649a particles at doses of 2.96×10^6 particles/ml and above (see Figure 8).

Relative Toxicity for ROFA, NIST 1649a, diesel particulates, and Mt. St. Helens Dust are calculated from the analysis of the linear regression data compiled in the Psi-Plot Program (V7.5)

Table 7. Calculation of Particle Equivalents for $SR_{.80}$*

Toxicant	$SR_{.80}$ (particles/ml)	r^2 (Regression)	Ratio to ROFA Dose
Mt. St. Helens Dust	13,838	.878	31.6
NIST 1649a	1,609	.964	3.67
Diesel Particulates	844	.971	1.93
ROFA	438	.942	-----

* Linear Regression Analysis was performed with the Psi-Plot software program. Particle concentrations for $SR_{.80}$ (Slope Ratio = 0.80) were obtained using the Dependent Variable Intercept Calculator.

Thus, ROFA is 1.93x more toxic than diesel particulates, 3.67 x more toxic than NIST 1649a, and 31.6x more toxic than Mt. St. Helens Dust in the *Tetramitus* Growth Inhibition Assay. Lower $SR_{.80}$ particle concentrations indicate **increased toxicity** because fewer particles are needed to produce a dose intercept value for that Slope Ratio ($SR_{.80}$) . The $SR_{.80}$ dose intercept value for cadmium chloride was 0.45 µg/mL. (see p 34).

Discussion:

ROFA and NIST 1649a were toxic in the *Tetramitus* Growth Inhibition Assay. Although the toxicity observed in the EPA Mouse Instillation Studies[4] used different assays, both ROFA and NIST 1649A were toxic in both mouse studies . Mt. St. Helens Dust exhibited low or no toxicity in both the *Tetramitus* and the mouse assays. These results confirm the utility of the *Tetramitus* Assay for rapid determination of air quality and settled dust contamination, following natural or terrorist toxic events. The ability to screen a large number of samples in a short period of time (24-30 hours) and the cost-effectiveness of the *Tetramitus* Assay, demonstrates the potential application for **environmental triage assessments**. Command decisions can designate potentially hazardous sites, pending further chemical and toxicity evaluations. Where cost considerations limit the number of specific chemical tests which can be performed, use of the *Tetramitus* Assay can serve to designate problem samples and assign priorities for further testing.

Assessment of the long-term health effects caused by exposure to WTC air and settled dust presents a daunting task to the research community. Use of *Tetramitus* toxicity data may contribute to a better understanding of possible risks. Evidence which supports the use of the *Tetramitus* Assay for assessment of long term health effects is:

1. The EPA has classified diesel particulates as a human carcinogen; *Tetramitus* flagellates exhibit a dose-dependent response to diesel particulates.

2. *Tetramitus* flagellates also exhibit a dose response to coal tar pitch condensate (CTP) used in the NIOSH mouse skin tumor studies[5]. The tumorogenic response in the mouse study was the same for CTP and benzo[a]pyrene, also classified as a carcinogen. The dose response in the *Tetramitus* Assay for CTP and benzo[a]pyrene also were similar.

3. *Tetramitus* flagellates exhibit a positive dose response to 26/27 DNA-damaging agents, which were tested.

4. Comparison of the *Tetramitus* dose-response data for five DNA-damaging agents to numerous other genotoxicity tests (See Appendix II) provides further circumstantial evidence for DNA damage being the cause of growth inhibition.

DNA damage is the postulated first step in the conversion of normal cells to cancer cells. DNA damage also weakens the immune system, and renders compromised individuals more susceptible to a wide spectrum of other diseases. Thus, *Tetramitus* exposure to mixtures of toxic agents may indicate potential long-term health effects.

The ability to assess mixtures provides additional information, which is not available by the evaluation of a list of individual toxicants. Furthermore, some "new" toxic agents may not be on the list of suspect agents. *Tetramitus* flagellates may be a cellular version of the canary in the coal mine paradigm.

References

1. 2000, Jaffe, R.L, The Tetramitus Assay, in Biomonitors and Biomarkers as Indicators of Environmental Change, Ed: Butterworth, F.M., A.Gunatilaka, and M.E. Gonsebatt pp.391- 425. Kluwar Academic/Plenum Publishers (New York)

2. 2000, Jaffe, R.L. Drinking Water Toxicity in New York City Reservoir and Tap Water Samples. Report to the New York City Council, January 14, 2000 (http://www.envirolab.com)

3 . 2000 , Jaffe, R.L., C.M. Ginn, and V.L. Keane. Tetramitus Toxicity Survey of Water Bodies in the Croton Watershed. (http://www.envirolab.com)

4. 2002 , Stephen H. Gavett, Najna Haykol-Coates, John K. McGee, Jerry W. Highfill, Allen D. Ledbetter and Daniel L. Costa. Toxicological Effects of Fine Particulate Matter Derived from the Destruction of the World Trade Center. United States Environmental Protection Agency, Office of Research and Development, National Health and Environmental Effects Research Laboratory, Research Triangle Park, NC. September, 2002. EPA/600/R-02/028.

5. 1995, Jaffe, R.L., Rapid Assay of Cytotoxicity Using Tetramitus Flagellates. Toxicology and Industrial Health 11: 543-558.

Appendix I

Description of the *Tetramitus* Assay and Data Management for Obtaining Toxicity Values

The Tetramitus Assay for Determination of Particle Toxicity

Tetramitus Assay Introduction:

A simple test for measuring cytotoxic agents using the flagellate phenotype of *Tetramitus rostratus* has been developed. The test measures dose-dependent inhibition of cell division by DNA-damaging agents and other toxicants. The *Tetramitus* Assay requires no animals or animal by-products. An additional attribute of the assay, which is useful for exposure monitoring and risk assessment, is the ability to measure whole particle toxicity without the need for prior solvent extraction and solvent-substitution procedures.

The assay is five to ten times more sensitive then standard EPA Whole Effluent Toxicity tests (WET Tests such as *Ceriodaphnia* and fat head minnow) and can be performed on non-sterile environmental samples[(1)]. A detailed protocol with standard operating procedures is described. Evaluation of growing cell populations in seed flasks prior to the actual performance of the test is predictive of test performance and, therefore, avoids the loss of valuable samples. The *Tetramitus* Assay will allow for frequent testing, thus permitting development of more accurate hazard assessments and comprehensive exposure models.

Tetramitus rostratus is a unicellular organism, which can exist as three distinct pheno-types: flagellate, ameba, or cyst. *Tetramitus* is estimated to have originated 1.0 to 1.2 billion years ago[(2)]. Single flagellates can be isolated and grown in liquid culture with bacteria as the only food source. Flagellates are quite stable (no amebae have been observed in more than 15,000 subcultures of flagellate populations reaching densities of up to 2 x 10^7 cells/mL). Because the *Tetramitus* flagellate is a particle feeder, the organism is useful for assessing whole particle cytotoxicity. The flagellate has a rigid cytoskeleton, four flagellae, and a gullet, which starts from the ventral depression and extends into the body of the cell (Fig. 1).

The assay measures dose-dependent inhibition of cell division by DNA-damaging agents. Recent studies have demonstrated the existence in *Tetramitus* of a 21.4 kb extrachromosomal DNA plasmid (r-DNA) which codes for the ribosomal RNA[(3)]. Minor sequence differences have been shown to cause drastic changes in the growth rate of *E. coli* cells harboring mutant rDNA plasmids[(4)]. Walsh has estimated the existence of 4,000 copies of rDNA amounting to 17% of the total cell DNA in single *Tetramitus* amebae[(5)] . The action of DNA-damaging agents on flagellates causes both decreased rates of cell division and a decrease in cell size.

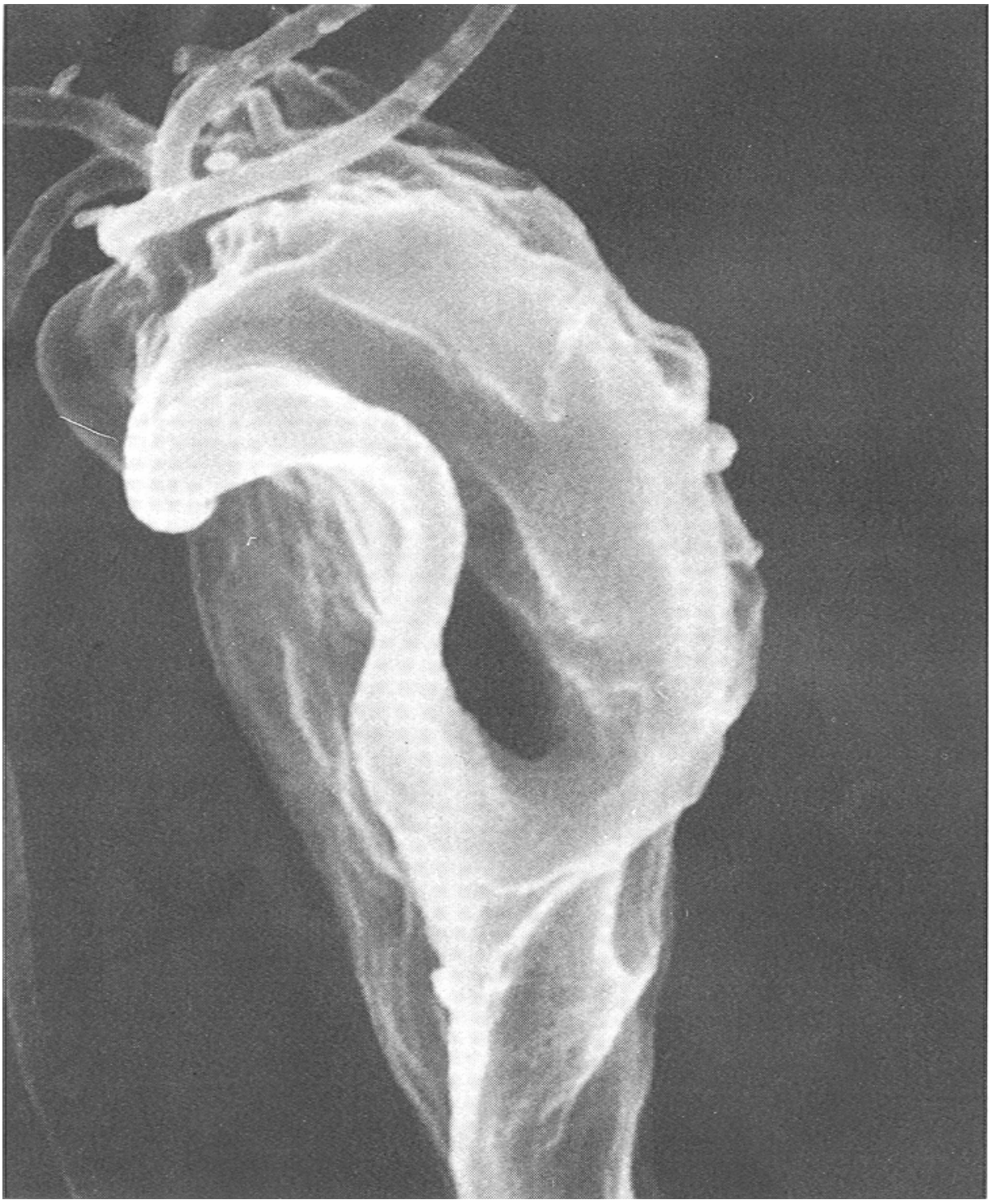


Figure 1. Scanning Electron micrograph of *Tetramitus* Flagellate [6]

The original description of methods for culturing flagellates and suggested test protocols has been published [7]. The following text describes the latest methods revisions.

Original Stock Cultures

Tetramitus flagellates are maintained in association with *Klebsiella pneumoniae* in YPP medium (0.05% Difco yeast extract and 0.05% Difco proteose peptone in distilled water) and grown in Corning 16 x 125 mm sterile polystyrene tissue culture tubes (25200). Flagellates inoculated from YPP medium into bacteria-buffer cultures usually took 5-6 subcultures before optimal growth conditions were observed (mean division time of 3.5 hours at 30°C). Cultures of *Tetramitus* flagellates can be obtained from the American Type Culture Collection (ATCC) located in Rockville, Md.

Standard Bacteria-Buffer Maintenance Cultures

Tetramitus flagellates are grown in MS-1 buffer containing a dense suspension of *Klebsiella pneumoniae*(Kp). MS-1 contains 0.1 mM KCl, 0.3 mM $CaCl_2$, 0.3mM NaH_2PO4, 0.0008% phenol red (pH indicator),and 1.4 mM $NaHCO_3$. The bicarbonate is added separately after autoclaving[8] . The original formulation of MS-1 called for inclusion of EDTA, which is now omitted in order not to interfere with toxicity testing of heavy metals.

Cultures of *Klebsiella* are grown overnight in a shaking water-bath at 35°C in 2.5% Oxoid #2 nutrient Broth (Unipath-CM67); 95 mL per 500 mL Nephlo flask (Bellco Glass 2581-14135). Teflon lined screw caps are used instead of the non-toxic rubber caps. Kp growth is monitored by determining the turbidity of the cultures in a Klett-Somerson nephelometer, using a red filter. The Kp are harvested by centrifugation at 2500 RCF for 10 minutes in Corning 50 mL sterile polypropylene tubes (25330-50). The Oxoid #2 supernatant is decanted, and the pellets are re-suspended in 40 mL of MS-1 by vigorous mixing with a Vortex Genie mixer. The Kp suspensions are re-centrifuged as above, the MS-1 supernatants are decanted and the pellets are re-suspended in fresh MS-1 (32 mL for each original 95 mL of Kp culture). The final suspension is referred to as Kp "soup" and 90 mL volumes (Kp suspension from 3 centrifuge tubes) are incubated in 350 mL baffled DeLong® flasks (Bellco 2510-00500) in a shaking water bath at 24°C @ 180 revolutions per minute. The New Brunswick Innova 3000 model has been found to be very reliable; all ETL's units are in use continuously without interruption. Our first unit has been in operation 24 hours a day for eleven years without a single malfunction.

Standard flagellate cultures were incubated in 125 mL baffled DeLong® flasks (Bellco 2510-00125) in 10 mL of Kp "soup" at 30°C @180rpm. Specially designed 125 mL flasks with 38 mm necks (Bellco Glass, special order as described in ETL/Bellco draft-specifications) are used for seed cultures in order to facilitate rapid pipetting of 50 µl aliquots into individual tubes (see below). Seed cultures, which are in log phase for at least three division cycles are optimal for toxicity testing. Cultures, which are about to enter into early stationary phase should not be used for testing; the dose-growth curves can exhibit regression data with r^2 values under 0.97. As the population enters stationary phase, the mean cell diameter decreases and any subculture derived from stationary phase cultures will exhibit an increase in cell diameter corresponding to the increase of its slope at that time point, thereby reflecting the growth status of the culture as it re-enters log phase (Figure 2). The mean cell diameter of any culture can be used to ascertain if that culture is in log phase, thus, serving as an objective quality control indicator for a given seed flask. Stock cultures of *Tetramitus* flagellates growing in Kp suspensions are routinely maintained. Thus, cultures are available for toxicity testing at desired temperatures within a 1-day advance time. Laboratories, which do not have the availability of a Beckman Coulter Multisizer or Z2, for size pattern determination, can use the oscilloscope patterns of the ZM as a means to evaluate size distributions. Precise size distributions can be obtained with the

ZM by manual recording of cell counts at specified size windows. Since this is labor intensive, we now use the three division-in-log-phase criterion for determination of seed culture acceptability for use in given toxicity tests.

A study was undertaken to determine the population heterogeneity of *Tetramitus* flagellates routinely grown in Kp suspension. 16 separate clones were isolated by dilution into individual tubes and were followed for a series of 20 subcultures in separate 1 mL Kp cultures. Table 1 summarizes the analysis of variance of the slopes of the 16 clones for series 5-8 subcultures growing at 30°C,

Table 1. Summary of Growth Rates of Tetramitus Flagellate Clones (Culture Series 5-8)
Slopes for each clone are measured from cell counts obtained at 3 time points. Statistical analysis include the mean slopes, coefficients of variance (CV), and the 95% and 99% confidence interval for the slopes of series 5-8 cultures. Flagellate cultures were maintained at 30°C.

Clone	Series 5	Series 6	Series 7	Series 8
TC-2	.0795	.0921	.0779	.0864
TC-3	.0901	.0781	.0735	.0907
TC-4	.0860	.0870	.0822	.0884
TC-5	.0778	.0909	.0764	.0939
TC-6	.0888	.0876	.0792	.0817
TC-7	.0759	.0936	.0760	.0806
TC-8	.0817	.0833	.0861	.0800
TC-9	.0738	.0761	.0783	.0792
TC-10	.0902	.0835	.0786	.0832
TC-11	.0796	.0722	.0721	.0803
TC-12	.0853	.0866	.0890	.0832
TC-13	.0776	.0904	.0848	.0845
TC-14	.0854	.0891	.0667	.0825
TC-15	.0829	.0863	.0812	.0844
TC-16	.0992	.0769	.0898	.0770
TC-17	.0919	.0807	.0948	.0751
MEAN SLOPE	.0841	.0847	.0804	.0832
Coefficient of Variance	8.09	7.46	8.97	5.88
95% Confidence	.0805 - .0877	.0813 - .0880	.0766 - .0843	.0806 - .0858
99% Confidence	.0791 - .0893	.0800 - .0893	.0751 - .0858	.0796 - .0868

Further observation of the population stability and homogeneity of the clones was demonstrated by measuring the mean cell diameters (MCDs) of the 16 clones during serial transfers of the series 18-20 cultures (Table 2). The mean cell diameter values differ from those shown in Figure 2, because of differences in the calibration between the Model Z2 and the Multisizer IIE and ZM. Our current calibration with the ZM indicates that the MCD value to be 9.4 - 9.6:. Note, that as the cell population enters stationary phase growth, the MCD decreases and the coefficient of variance increases

Table 2. Mean Cell Diameters (MCD) of *Tetramitus* Clones during serial transfer (30°C) . MCDs were determined with a Beckman Coulter Z2 Counter.

Clone	Mean Cell Diameter (µm)	Mean Cell Diameter (µm) (Late Log-SP) Series 18	Mean Cell Diameter (µm)	Mean Cell Diameter (µm)	Mean Cell Diameter (µm) (20°C)
TC-2	11.07	10.06	10.49	10.66	10.81
TC-3	11.21	9.75	10.87	10.72	10.88
TC-4	10.84	9.51	10.80	10.86	11.16
TC-5	10.85	9.46	10.82	10.75	11.09
TC-6	10.68	9.47	10.82	10.48	10.89
TC-7	10.78	10.73	10.71	10.74	10.94
TC-8	10.59	10.09	10.28	10.72	10.81
TC-9	10.84	10.88	10.92	11.03	11.02
TC-10	11.22	9.34	10.83	10.81	10.88
TC-11	10.83	10.78	10.69	10.48	10.81
TC-12	10.53	9.12	10.44	10.40	10.62
TC-13	10.64	9.69	10.68	10.61	10.79
TC-14	10.79	10.73	10.68	10.53	11.00
TC-15	10.71	9.82	10.36	10.59	NA
TC-16	10.77	9.05	10.64	10.69	10.81
TC-17	10.43	10.51	10.68	10.54	10.70
MEAN	10.80	9.94	10.67	10.66	10.88
Coefficient of Variance	2.04	6.24	1.76	1.52	1.32

These data demonstrate the stability and reproducibility of *Tetramitus* flagellate growth in serial laboratory cultures.

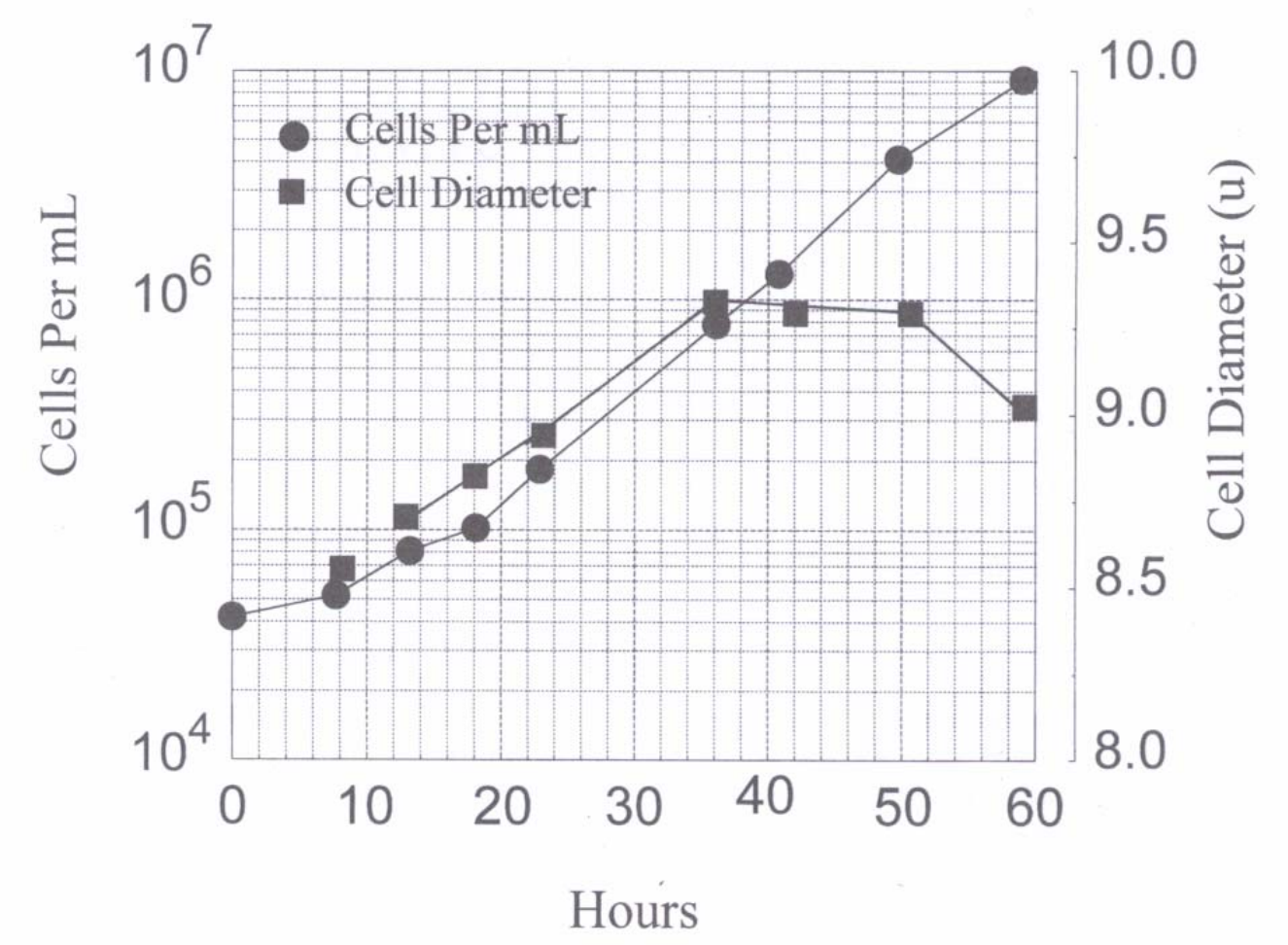


Figure 2. Growth of *Tetramitus* Flagellates at 30°C. The ■ symbols indicate the mean cell diameters as determined by the statistics program of the Multisizer IIE. When the cells are in log phase, the mean cell diameter values display a constant value.

Counting

Cell concentrations were determined with the use of either a model ZM Coulter Counter or a Multisizer IIe (Beckman-Coulter Electronics, Miami, Florida) using a 100 µ aperture tube. 0.2 mL aliquots were transferred to Folin-Wu tubes containing 30 mL of electrolyte (0.4% NaCl [w/v] in distilled water). The volume was adjusted to 35.0 mL by adding saline from a plastic wash bottle to the etched 35 mL volume line of the Folin-Wu tubes. The contents of each tube were agitated using a Vortex-Genie mixer, aliquots were transferred to Coulter disposable counting cuvettes and 2 counts were determined at threshold settings of 10-99.9; current, 400 mA; attenuation, 4; preset gain, 1; and manometer selection, 500ul for the ZM. The narrow channel option with lower channel = 6.03 µ and upper channel = 15.03µ settings are used for the Multisizer. This method of counting has been found to be extremely reliable; the correlation coefficients of the growth curves are usually 0.998 or higher. One correlation coefficient of a four-point growth curve was 0.999999. The precision of the Coulter

Counters, both the Multisizer IIe and the ZM, over the course of 10 years of research experience has been a constant ingredient in the production of a data base containing control and dose growth regression curves with high correlation coefficients.

Standard hemacytometer counting methods can be employed by pipetting 100 µl of sample into a 12 x 75 mm polystyrene test tube (Fisher 14-961-10) containing 10 µl of Lugol's iodine. After vortex-mixing, aliquots are transferred to a counting chamber. 4 counts of 100 + are obtained and the cell concentration is calculated by multiplying the average count by 1.1(to compensate for the 10 µl of Lugol's iodine). The larger area of the hemacytometer chamber can be employed using an additional dilution multiplier of 1.1×10^3. The lowest flagellate concentration, which accurately, can be measured by this method, would be 1×10^5 cells/mL

Data Management

The growth of each control (MS-1) and dose culture were recorded by entering the cell counts into a Lotus™ spread-sheet (Table 3), which was modified to list the time of sampling, elapsed time, 2-4 coulter counter determinations, average cell concentration, and the log of the cell concentration. The summary table lists the regression calculations. Templates for toxicity tests employing 4 – 7 doses also contained macros, which provided summary sheets listing the slope-ratio calculations for each dose.

The data from the Lotus™ spreadsheets were transferred to PSI-Plot (Poly Software, Pearl River, NY) spreadsheets in order to produce growth and dose-response graphics

Table 3. Data entry into Lotus 123 ® Spreadsheets. Each spreadsheet contains a series of tests: Test 1 is the control culture (MS-1 or 0-dose), the cultures with increasing doses are labeled Test 2, Test 3, etc. The slopes, r^2 values and the slope ratios are automatically calculated by a series of macro commands and are listed in the summary sheet:

Test 1 Control (MS-1)
Dilution Factor : 1/350

	Initial	**1**	**2**
Time of reading	13:21:89	4:38:31	14:07:33
Elapsed Time	0.00	15.28	22.70
Coulter Counts			
Reading 1	43	948	10966
Reading 2		975	11360
Average	43	961.5	11163
Log Cells/mL	4.1795	5.5270	6.5918
Cells/mL	1.512×10^4	3.365×10^5	3.907×10^6

EFFECT OF CONCENTRATION **(Summary Sheet for 3 doses)**

Concentration	Slope	r^2	Slope Ratio
MS-1 (Control)	0.0869	0.9999	1
20%	0.0811	0.9931	0.933
50%	0.0749	0.9939	0.862
90%	0.0645	0.9969	0.740

Cultures for Toxicological Studies

Individual Toxicants:

Toxicological studies are carried out in 17 x 100 mm Falcon (35-2057) sterile, disposable polystyrene tubes; final volumes are 1.0 mL/tube. For organic toxicants, 10 µL aliquots of serial dilutions of toxicant dissolved in dimethylsulfoxide (DMSO) are added to 990 µL aliquots of flagellate cultures in order to obtain a series of dose-culture tubes. 10 µL of DMSO is used for the 0-dose or control tube. Inorganic toxicants are dissolved and diluted in MS-1. Some organics which are not soluble in DMSO, such as benzo[a]pyrene are dissolved in cyclohexane.

Environmental Samples:

Whole Effluent and whole particle testing are conducted in 1.0 mL final volumes according to the dilution matrix described in Table 4. The protocol has been modified in order to permit allocation of 90% of the volume of the test cultures for delivery of the sample. In order to reconstitute the whole effluent or water samples in MS-1 buffer; 60 µL of solution A, 30 µL of 0.1M $CaCl_2.2H_2O$, and 100 µL of 0.1M $NaHCO_3$ are added to 10 mL of neat water sample. Solution A contains 10 mL of 0.1 M $NaHPO_4$, 10 mL of .5% phenol red solution (Sigma P-0290), and 3 mL of 0.1M KCl.

Table 4. Schedule of components for testing either whole effluent water samples, or whole particle suspensions. The units of measurement for whole water dilutions are % Effluent; and Particles/mL for particle suspensions.

Whole Effluent Whole Particle	MS-1	Flagellates (Seed Flask)	Kp Suspension (20X)
0 µL	900 µL	50 µL	50 µL
100 µL	800 µL	50 µL	50 µL
200 µL	700 µL	50 µL	50 µL
500 µL	400 µL	50 µL	50 µL
900 µL	--------	50 µL	50 µL

20X Kp Preparation and Seed Cultures:

The 20 X Kp (*Klebsiella pneumonia*) suspension (Table 4) is obtained by re-centrifuging the Kp suspension (see above) and re-suspending the Kp in 1/20 of the original volume (ex: re-suspend the pellet obtained from 100 mL of Kp soup in 5.0 mL of MS-1). The starting flagellate concentration in the 1.0 mL of test culture will be 1/20 of the seed culture. Aliquots of whole water samples also may be filtered through membrane filters of known pore size in order to determine the toxicity of filtrates, which are selected for exclusion of particles of specific sizes.

The sequence for the test is:

1) Set up the seed flask culture to contain 2.0 x 10^5 cells per mL (572 counts determined by Coulter Multisizer or ZM) at the anticipated time of delivery. 50 µL delivered to each tube would result in a starting concentration of 1 x 10^4 cells per mL. The slope of the dose response curves for individual toxicants, whole effluents, and water concentrates is steeper at lower starting flagellate concentrations (Figure 3). Consequently, all tests are standardized for this starting concentration. The usual slopes of log phase cultures growing at 30°C, range between .082-.093 (log cell concentration per hour). The mean division time is about 3.7 hours and the time required to grow 1 log is 11.5 hours (ex: 1 x 10^4 – 1 x 10^5 cells/ml). Seed cultures can be diluted with Kp soup several hours before tests commence in order to ensure the 1 x 10^4 per mL starting concentration.

2) Test components are delivered into each Falcon 17 mm tube in the following sequence:

MS-1, 20 X Kp, particles or whole effluent, vortex and place in water bath.

3) Count cells in seed flask and then deliver 50 µL aliquots as rapidly as possible. Use the recorded time of seed flask determination and designate the starting flagellate concentration as 1/20 of the recorded seed flask, Coulter count value. Delivery of 10 x 5 aliquots (10 tests with 4 dilutions + control) usually takes 12-15 minutes.

4) Set up Test 1 spreadsheet (separate file label) with recorded time and calculated, starting Coulter counts for the control culture, then copy these values to the 4, 5, or 6 dose-culture spread sheet cells; save the file. Copy this file to nine separate files, using the "Save As" Tab in the spreadsheet file menu. Assign different file labels for different tests. The spreadsheets can be formatted at any time prior to the first sampling time.

5) Count each test series at three subsequent sampling times, usually at 20-22 hours, 29-32 hour, and 40-44 hours. Enter the time of sample taking and the Coulter counts in each Lotus spreadsheet. The summary page will automatically list the calculated values of the slopes, r^2 values and the slope-ratios for each dilution. These values can be exported to a statistics program (PSI-Plot, Excel, Statmost, etc.) to produce graphics of the dose-response curves.

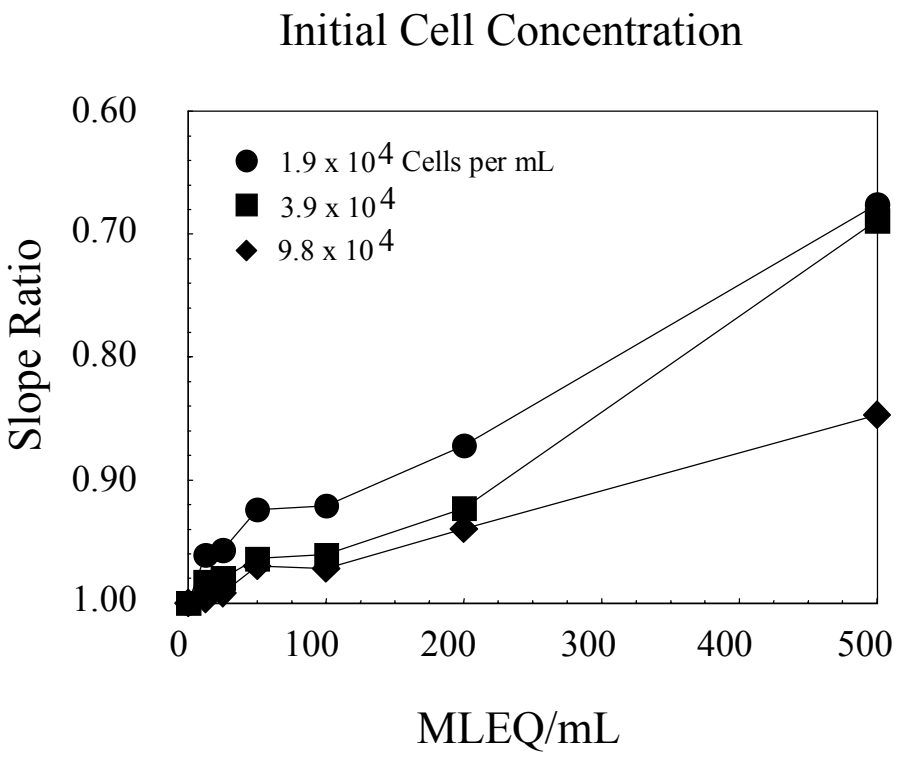


Figure 3. The effect of initial cell concentration on the dose response of a Netherlands water concentrate. The slope ratio x concentrate dose values was obtained for each of the starting flagellate concentrations. The regression curves for each starting cell concentration describe the increased sensitivity of the test at lower starting cell concentrations (decreasing slope ratios are a measure of increasing cell division inhibition and indicate increased toxicity).

Determination of the storage time stability of particles both in whole water and in suspension after filter preparation will be valuable for scheduling the elapsed times after sample collection for optimal performance of tests.

Obtaining Dose Response Curves

- **Plot growth curves for each dose-culture**
- **Calculate the slope for each dose-growth curve**
- **Calculate the slope ratio for each dose:**

  slope of dose culture

  slope of control culture

- **Plot Dose Response Curve**

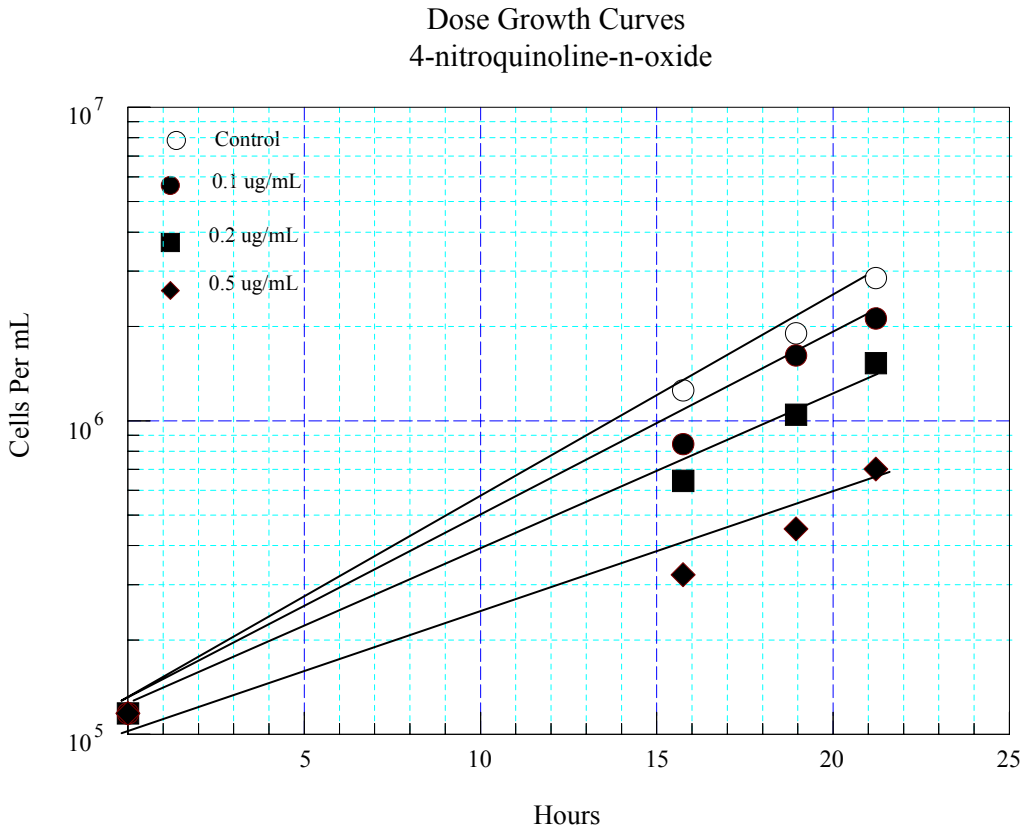


Figure 1. Growth of Tetramitus Flagellates at different concentrations of 4-nitroquinoline-n-oxide.

Dose	Slope (1) Growth Curves	r^2 (*) Growth curves	Slope Ratio (Relative Growth)
0 µg/mL	.0653	.9997	1.0
0.1 µg/mL	.0591	.9942	.906
0.2 µg/mL	.0512	.9929	.784
0.5 µg/mL	.0338	.9591	.517

(1) Log of cell concentration per /hour

(*) correlation coefficient. Perfect correlation (all points on a straight line) is 1.0

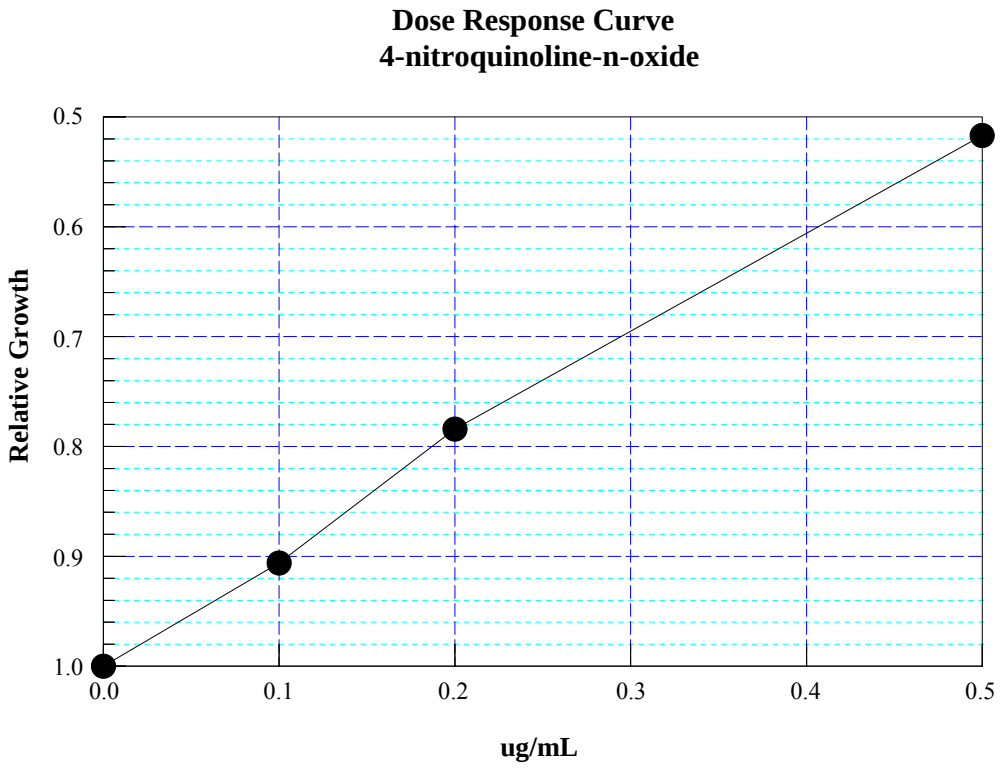


Figure 2. **Dose Response curve for 4-nitroquinoline-n-oxide. Lower Relative Growth (Slope Ratio) indicates increased toxicity.**

Appendix I References

1. Jaffe, R.L, (2000) The Tetramitus Assay, in Biomonitors and Biomarkers as Indicators of Environmental Change, ed: Butterworth, F.M., A.Gunatilaka, and M.E. Gonsebatt pp.391- 425. Kluwar Academic/Plenum Publishers (New York).

2. Sagan, L. (1967) On The Origin of Mitosing Cells. J. Theoret. Biol. 14: 224-274.

3. Clark, C. G. and G. A. M. Cross. (1988). Circular Ribosomal RNA Genes Are A General Feature of Schizopyrenid Amoebae. J. Protozool. 35: 326-329.

4. Steen, R., D. K. Jemiolo, R. M. Skinner, J. J. Dunn, and A. E. Dahlberg. (1986). Expression of Plasmid-Coded Mutant Ribosomal RNA in E. coli:Choice of Plasmid Vectors and Gene Expression Systems. Progress in Nucleic Acid Research and Molecular Biology 33: 1-18.

5. Walsh, C. 1996, personal communication.

6. Balamuth, W., P.C. Bradbury, and F.L. Schuster (1983) Ultrastructure of the amoeboflagellate *Tetramitus rostratus*. J. Protozool. 30: 445-455.

7. Jaffe, R.L. (1995) Rapid Assay of Cytotoxicity using *Tetramitus* Flagellates. Toxicology and Industrial Health 11: 543-558.

8. Fulton, C. (1970). Amebo-flagellates as research partners: the laboratory biology of *Naegleria* and *Tetramitus*. Methods Cell Physiol. 4: 341-476.

Appendix II.

Reference Toxicants in the Tetramitus Assay

A. Dose Response to 8 Reference Toxicants

B. Comparison of 5 Reference Toxicants to other Genotoxicity Tests

C. Range of Flagellate Growth Inhibition Response

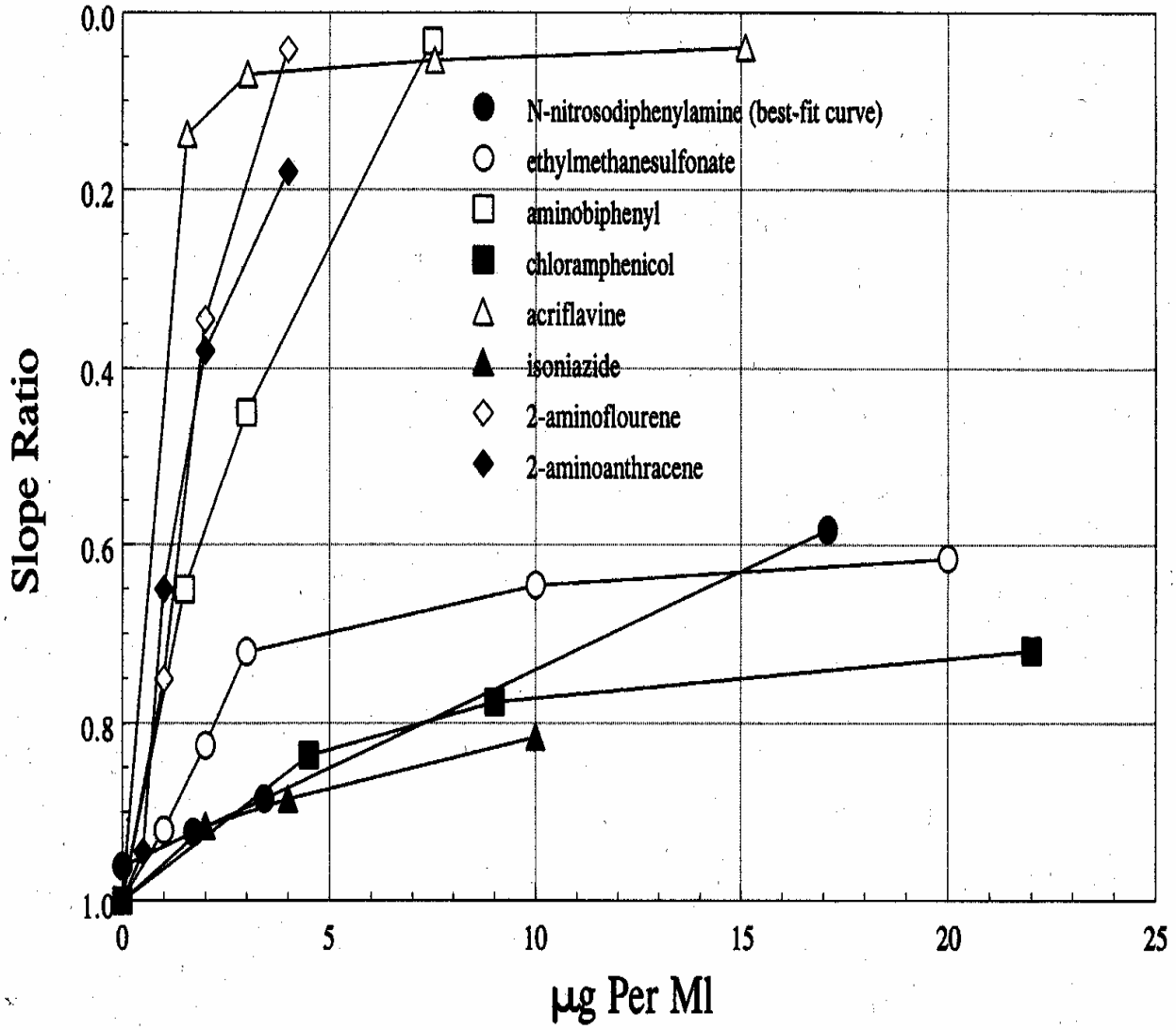
D. Dose Response to MX

Major disinfection byproduct of drinking water chlorination

E. National Institute of Standards (NIST) Standard Reference Materials

Diesel Particulate Matter NIST SRM # 2975

Coal Fly Ash NIST SRM # 2689



Toxicant Concentration

Figure A. Dose Response of Tetramitus Flagellates to eight reference toxicants. Lower Slope Ratios (**Relative Growth**) indicates increased toxicity.

B. Comparison of Tetramitus Assay to other Genotoxicity Tests for 5 Reference Toxicants

TABLE 3. Comparison of *Tetramitus* Test to Ames/*Salmonella* and Other Genotoxicity Tests (Waters et al., 1994)

Mutagen	*Tetramitus* test dose (µg/ml) slope ratio = 0.8	Ames/*Salmonella* test LED* (µg/ml)		Other tests LED* (µg/ml)	
Cadmium chloride	0.45	8	(TA 102)	0.01–1.1	CIC
		56–1120	(TA 100)	0.66–3.66	AVA
		56–610	(TA 1535)	0.37–9.0	SPM
		150–610	(TA 98)	1.0–4.3	DLM
Aminobiphenyl	1.2	2–62	(TA 98)	5	G5T
		1–62	(TA 1538)	6.3	GIH
		5–125	(TA 1535)	10	G9H
		0.1–1250	(TA 100)		
Methyl glyoxal	3.5	1.25	(TA 104)	7	SIC
		2–5	(TA 100)	30	GCL
		2–30	(TA 102)	36	G9H
Methyl yellow	7.5	2–50	(TA 98)	6.25	G5T
		5–2500	(TA 1538)	16	GIA
				22.5	SIC
2-Nitrofluorene	22	0.15–50	(TA 1538)	2–4	G5T
		0.3–50	(TA 98)	2.1	SIC
		0.5–17	(TA 1537)	5–167	ECW
		1.7–1250	(TA 100)	20	TCS
		5–167	(TA 1535)		

*LED, lowest effective dose (ranges include values reported from multiple citations).
Other test codes: AVA, aneuploidy, animal cells *in vitro*; CIC, chromosomal aberrations, Chinese hamster cells *in vitro*; DLM, dominant lethal test, mice; ECW, *E. coli*, WP2 uvrA, reverse mutation; GIA, gene mutation, animal cells *in vitro*; GIH, gene mutation, human cells *in vitro*; G5T, mouse lymphoma cells, TK locus; G9H, gene mutation, Chinese hamster lung V-79 cells; GCL, Chinese hamster lung cells exclusive of V79; SIC, sister chromatid exchange, Chinese hamster cells *in vitro*; SPM, sperm morphology, mouse; TCS, cell transformation, Syrian hamster embryo cells, clonal assay.

Waters, N., F. Stack, M. Jackson, W. Lohman, F. Lohman, D. McGregor, and H. Vainio. (1994) EPA/IARC Genetic Activity Profiles, Computer Program (Release 4.06), Research Triangle Park, NC.

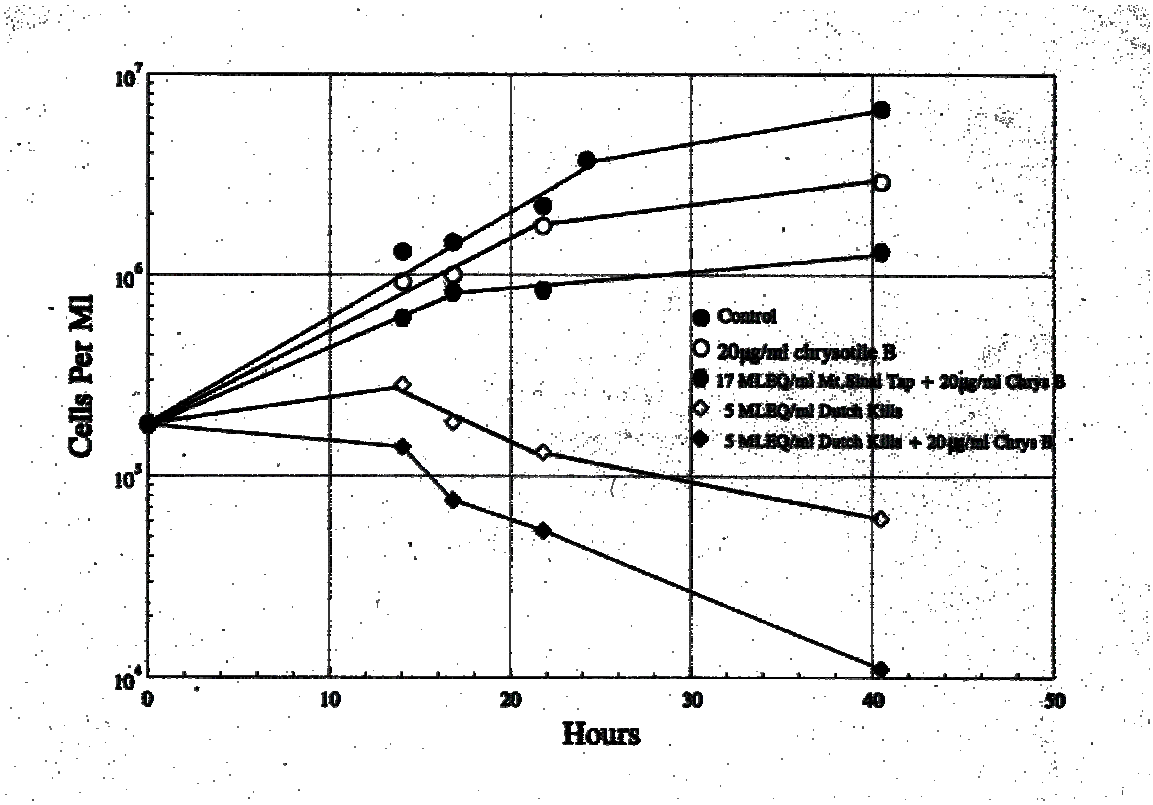
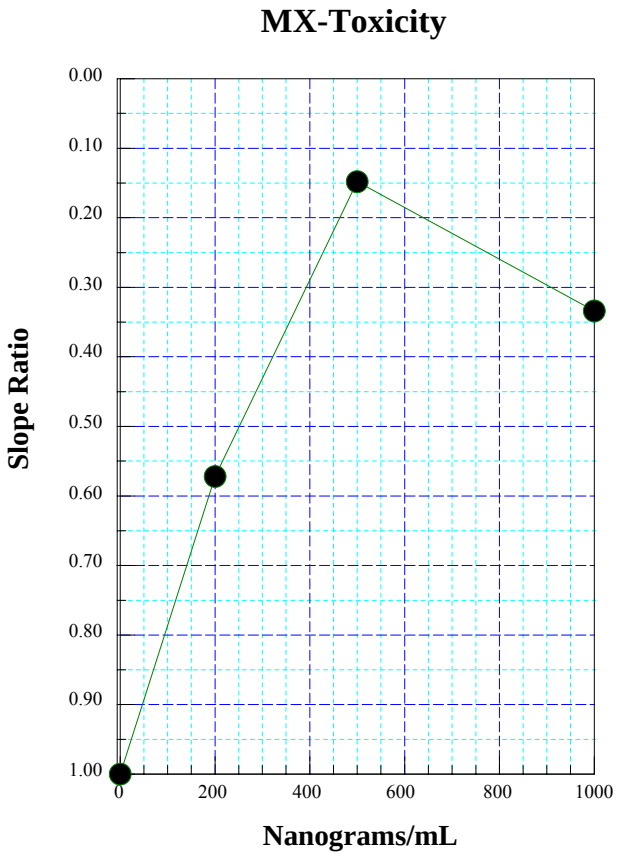


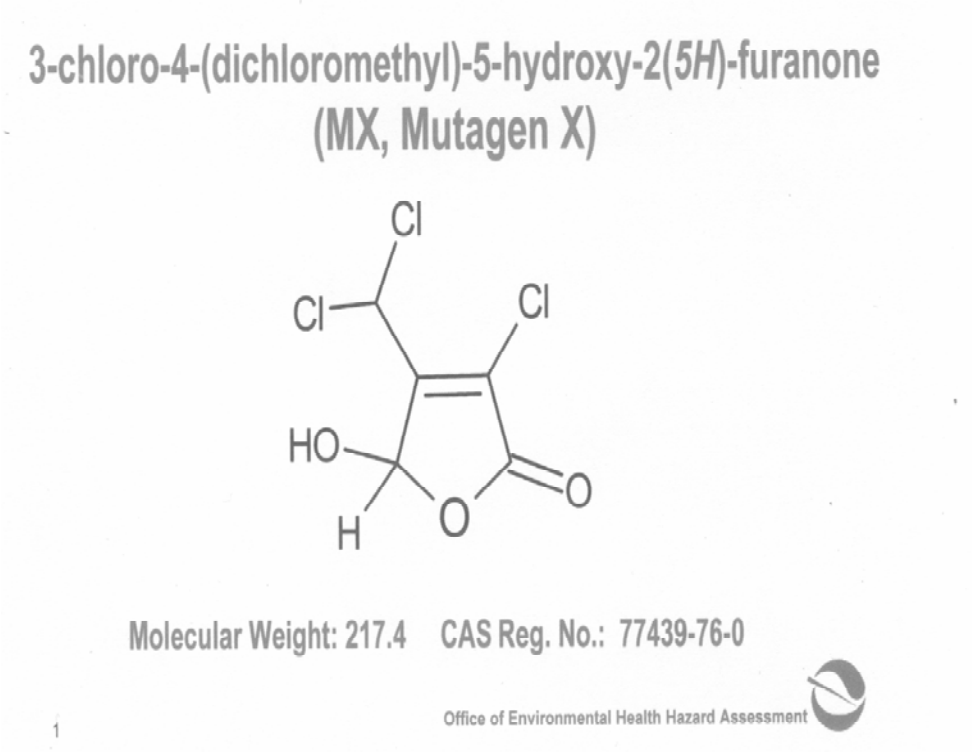
Figure C. Growth of Tetramitus Flagellates in Mt. Sinai Tap Water and Dutch Kills water concentrates. Note the synergistic effect of water concentrate + chrysotile asbestos. Flagellate growth only in Mt. Sinai Water Concentrate is similar to the growth of the control culture.

The range of Growth Responses can exhibit:

- no toxicity (same growth rate as control cultures; slope ratio = 1)
- Decrease in relative growth rate (Slope ratio values decrease from value of 1.0; a Slope Ratio of 0.9 indicates that the cells are growing at 90% of the controls).
- Complete Growth Inhibition
- Decrease in cell number (cell death)

Figure D Tetramitus MX Toxicity





MX: Summary

- **Animal evidence of carcinogenicity:**
  - Induction of tumors at multiple sites in both male and female rats following treatment via drinking water for 2 years
- **Other relevant evidence:**
  - Extensive genotoxicity evidence both *in vitro* and *in vivo*
  - Suggestive evidence that MX may induce cellular proliferation or promote tumors in some tissues

E. National Institute of Standards (NIST) Standard Reference Materials (SRM)

Diesel Particle Matter NIST SRM # 2975

Coal Fly Ash NIST SRM # 2689

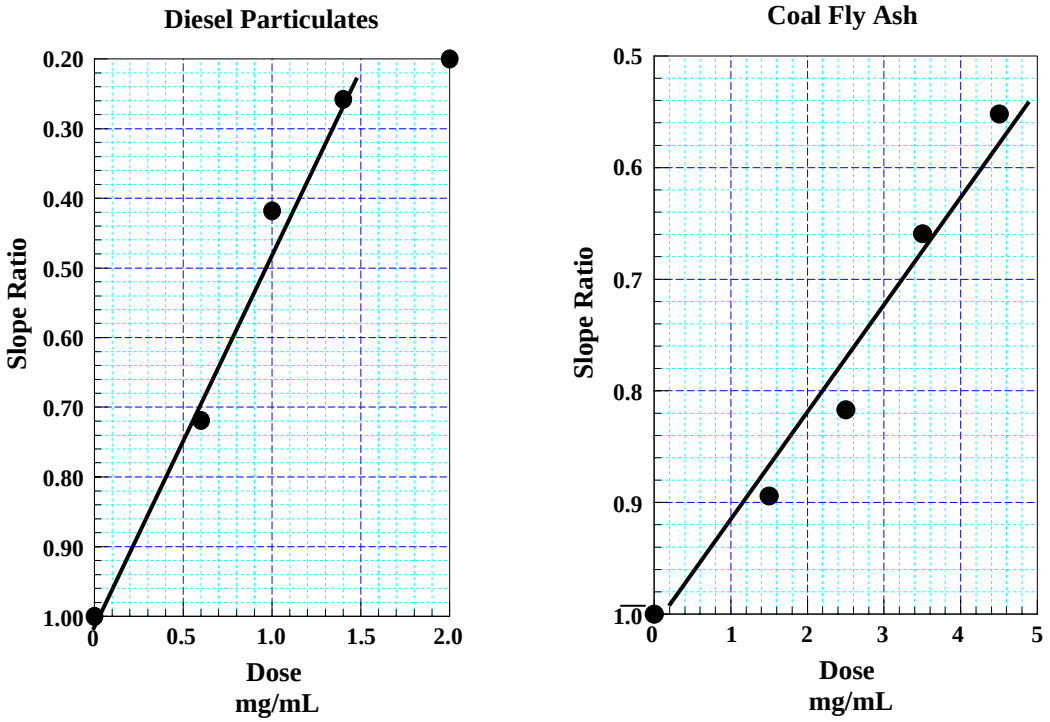


Figure E. Dose Response of Tetramitus flagellates to Diesel Particles and Coal Fly Ash. Lower Slope Ratios (Relative Growth) indicate greater toxicity.

Appendix III.

Comparison of *Tetramitus* Assay to EPA WET Tests

The Michigan Split-Sample Study compared the *Tetramitus* Assay to standard EPA whole effluent assays (WET tests) , *Ceriodaphia* and the fat head minnow, for a 2^0 sewage plant, a pharmaceutical plant, and an automobile plant effluent (Jaffe,RL, Sweet ,LI, and Meier,PG). Table 1 illustrates that the *Tetramitus* Assay was at least five times more sensitive than the standard WET test. Only the pharmaceutical plant produced a NOEC (no observable effect concentration) of 60% effluent using standard WET tests; both the 2^0 sewage and auto manufacturing plant effluents had NOEC's of 100%. The NOEC's for the *Tetramitus* assays were 20 -40%. All the assays were conducted with starting flagellate concentrations of 1.0×10^5 cells per mL. Figure 9-11 is a graphic comparing WET Tests performed on different effluents. One series using a starting cell concentration of 1×10^4 produced a dose response curve, which was 2.5 x more sensitive (based on comparison of the slope-ratio intercept of 0.90). Similar increased sensitivity also was observed on a Netherlands water concentrate (see above). Whole effluent samples also may be filtered through 0.45µ pore size membrane filters. The filtered whole effluents can be tested in order to observe the effect of particle contribution (greater than 0.45µ) on the unfiltered whole effluent dose response curves. In addition, particle toxicity can be directly measured by testing concentrated particle suspensions (obtained by centrifugation)

Table 1. Comparison of the *Tetramitus* Assay to , EPA WET Tests using *Ceriodaphnia* ,and the Fathead minnow.

Source of Effluent	WET Test Organism	NOEC(1)	LOEC(2)
Pharmaceutical Plant	*Tetramitus*	20% effluent	40% effluent
	Ceriodaphnia	60% effluent	80% effluent
	Fathead minnow	60% effluent	80% effluent
Auto-Manufacturing Plant	*Tetramitus*	20% effluent	40% effluent
	Ceriodaphnia	100% effluent	NA
	Fathead minnow	100% effluent	NA
2^o Sewage Plant	*Tetramitus*	20% effluent	40% effluent
	Ceriodaphnia	100% effluent	NA
	Fathead minnow	100% effluent	NA

(1) **NOEC** - No Observable Effect Concentration.
(2) **LOEC** - Lowest Observable Effect Concentration.
(3) **NA** - Not Available.

Reference:

1998 Jaffe, R.L., L.I. Sweet and P.G. Meier. Whole Effluent Toxicity Testing using Tetramitus Flagellates. Abstract, 4th Annual NAC SETAC Meeting, Saratoga Springs, N.Y.

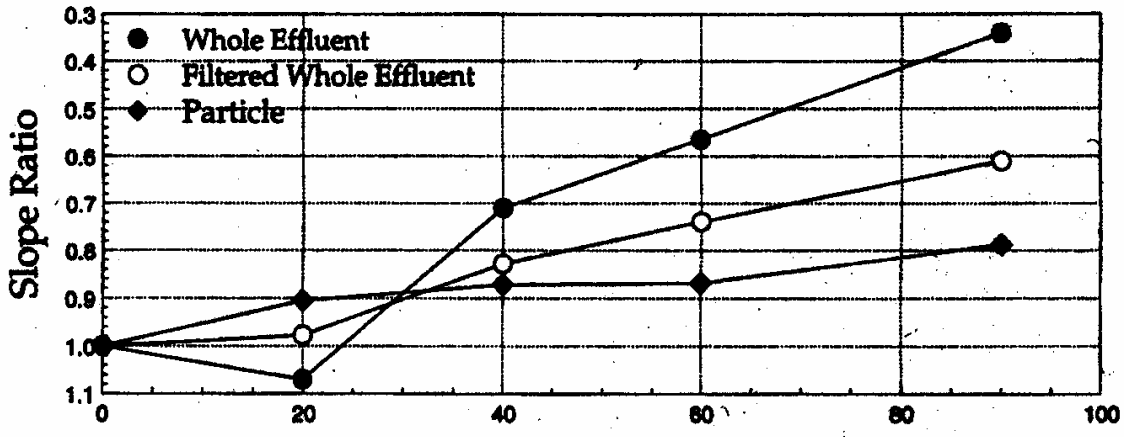


FIGURE 9. Dose response curves of components in an effluent from an auto manufacturing plant. The whole water toxicity is the sum of the filtrate + particulate toxicity.

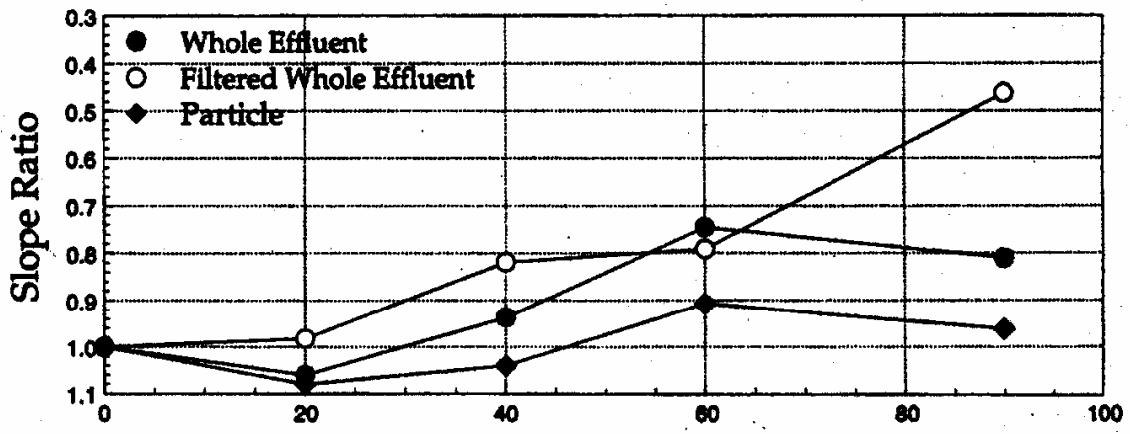


FIGURE 10. Dose response curves of components from a secondary sewage treatment plant effluent. Particle interactions above 60% effluent and 60 Part-MLEO/mL cause flattening of both whole effluent and particle dose response curves while the filtered whole effluent curve remains linear.

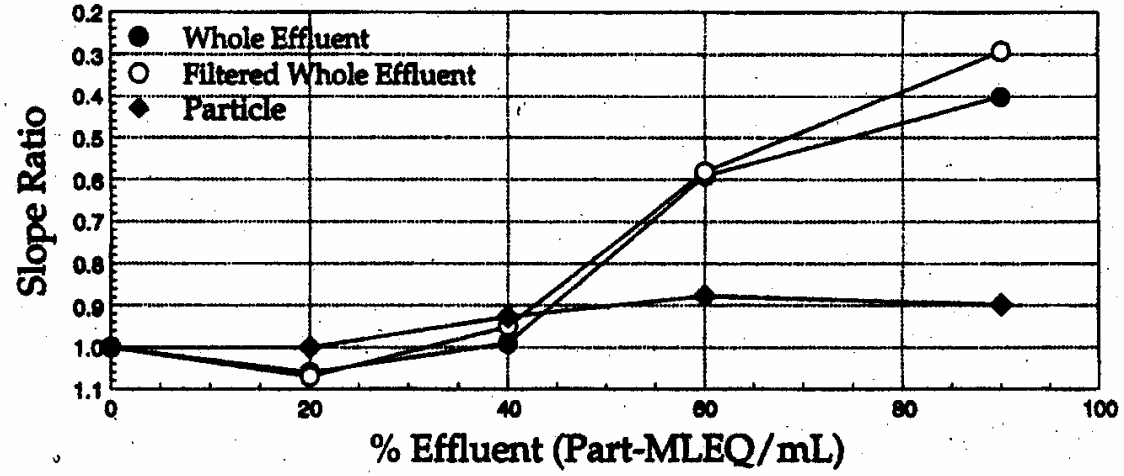


FIGURE 11. Dose response curves of components from a pharmaceutical plant effluent. Whole Effluent and Filtered Whole Effluent curves are similar.